



## Microbiota stability in healthy individuals after single-dose lactulose challenge-A randomized controlled study

Wotzka, Sandra Y ; Kreuzer, Markus ; Maier, Lisa ; Zünd, Mirjam ; Schlumberger, Markus ; Nguyen, Bidong ; Fox, Mark ; Pohl, Daniel ; Heinrich, Henriette ; Rogler, Gerhard ; Biedermann, Luc ; Scharl, Michael ; Sunagawa, Shinichi ; Hardt, Wolf-Dietrich ; Misselwitz, Benjamin

**Abstract:** BACKGROUND AND AIMS Lactulose is a common food ingredient and widely used as a treatment for constipation or hepatic encephalopathy and a substrate for hydrogen breath tests. Lactulose is fermented by the colon microbiota resulting in the production of hydrogen (H<sub>2</sub>). H<sub>2</sub> is a substrate for enteropathogens including Salmonella Typhimurium (S. Typhimurium) and increased H<sub>2</sub> production upon lactulose ingestion might favor the growth of H<sub>2</sub>-consuming enteropathogens. We aimed to analyze effects of single-dose lactulose ingestion on the growth of intrinsic Escherichia coli (E. coli), which can be efficiently quantified by plating and which share most metabolic requirements with S. Typhimurium. METHODS 32 healthy volunteers (18 females, 14 males) were recruited. Participants were randomized for single-dose ingestion of 50 g lactulose or 50 g sucrose (controls). After ingestion, H<sub>2</sub> in expiratory air and symptoms were recorded. Stool samples were acquired at days -1, 1 and 14. We analyzed 16S microbiota composition and abundance and characteristics of E. coli isolates. RESULTS Lactulose ingestion resulted in diarrhea in 14/17 individuals. In 14/17 individuals, H<sub>2</sub>-levels in expiratory air increased by 20 ppm within 3 hours after lactulose challenge. H<sub>2</sub>-levels correlated with the number of defecations within 6 hours. E. coli was detectable in feces of all subjects (2 x 10<sup>2</sup>-10<sup>9</sup> CFU/g). However, the number of E. coli colony forming units (CFU) on selective media did not differ between any time point before or after challenge with sucrose or lactulose. The microbiota composition also remained stable upon lactulose exposure. CONCLUSION Ingestion of a single dose of 50 g lactulose does not significantly alter E. coli density in stool samples of healthy volunteers. 50 g lactulose therefore seems unlikely to sufficiently alter growth conditions in the intestine for a significant predisposition to infection with H<sub>2</sub>-consuming enteropathogens such as S. Typhimurium ([www.clinicaltrials.gov](http://www.clinicaltrials.gov) NCT02397512).

DOI: <https://doi.org/10.1371/journal.pone.0206214>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-158476>

Journal Article

Published Version



The following work is licensed under a Creative Commons: Attribution 4.0 International (CC BY 4.0) License.

Originally published at:

Wotzka, Sandra Y; Kreuzer, Markus; Maier, Lisa; Zünd, Mirjam; Schlumberger, Markus; Nguyen, Bidong; Fox, Mark; Pohl, Daniel; Heinrich, Henriette; Rogler, Gerhard; Biedermann, Luc; Scharl, Michael; Sunagawa, Shinichi; Hardt, Wolf-Dietrich; Misselwitz, Benjamin (2018). Microbiota stability in healthy individuals after single-dose lactulose challenge-A randomized controlled study. PLoS ONE, 13(10):e0206214.  
DOI: <https://doi.org/10.1371/journal.pone.0206214>

RESEARCH ARTICLE

# Microbiota stability in healthy individuals after single-dose lactulose challenge—A randomized controlled study

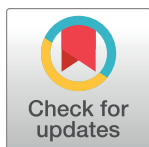
Sandra Y. Wotzka<sup>1</sup>, Markus Kreuzer<sup>1</sup>, Lisa Maier<sup>1</sup>, Mirjam Zünd<sup>1\*</sup>, Markus Schlumberger<sup>1</sup>, Bidong Nguyen<sup>1</sup>, Mark Fox<sup>2</sup>, Daniel Pohl<sup>3</sup>, Henriette Heinrich<sup>2,3</sup>, Gerhard Rogler<sup>3</sup>, Luc Biedermann<sup>3</sup>, Michael Scharl<sup>3</sup>, Shinichi Sunagawa<sup>1†\*</sup>, Wolf-Dietrich Hardt<sup>1†\*</sup>, Benjamin Misselwitz<sup>3†\*</sup>

**1** Institute of Microbiology, D-BIOL, ETH Zürich, Zürich, Switzerland, **2** Abdominal Center, Gastroenterology, St. Claraspital, Basel, Switzerland, **3** Department of Gastroenterology, University Hospital Zurich (USZ), and Zurich University, Zurich, Switzerland

\* These authors contributed equally to this work.

† These authors also contributed equally to this work.

\* [wolf-dietrich.hardt@micro.biol.ethz.ch](mailto:wolf-dietrich.hardt@micro.biol.ethz.ch) (WDH); [shinichi.sunagawa@micro.biol.ethz.ch](mailto:shinichi.sunagawa@micro.biol.ethz.ch) (SS); [benjamin.misselwitz@usz.ch](mailto:benjamin.misselwitz@usz.ch) (BM)



## OPEN ACCESS

**Citation:** Wotzka SY, Kreuzer M, Maier L, Zünd M, Schlumberger M, Nguyen B, et al. (2018) Microbiota stability in healthy individuals after single-dose lactulose challenge—A randomized controlled study. PLoS ONE 13(10): e0206214. <https://doi.org/10.1371/journal.pone.0206214>

**Editor:** Christopher Staley, University of Minnesota Twin Cities, UNITED STATES

**Received:** April 9, 2018

**Accepted:** October 9, 2018

**Published:** October 25, 2018

**Copyright:** © 2018 Wotzka et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** Sequences obtained for this study are available via the ENA database under the accession number: PRJEB28417.

**Funding:** This work was supported by a Seed grant from Hochschulmedizin Zurich (to GR and WDH), a grant from ETH Zurich (ETH-33 12-2; to WDH, partially covering the salary of SYW), a grant from the Novartis Foundation (#15C181; to WDH, partially covering the salary of SYW), the Swiss National Science Foundation (SNF 310030\_153074, 3130030B\_173338 and

## Abstract

### Background and aims

Lactulose is a common food ingredient and widely used as a treatment for constipation or hepatic encephalopathy and a substrate for hydrogen breath tests. Lactulose is fermented by the colon microbiota resulting in the production of hydrogen (H<sub>2</sub>). H<sub>2</sub> is a substrate for enteropathogens including *Salmonella* Typhimurium (*S. Typhimurium*) and increased H<sub>2</sub> production upon lactulose ingestion might favor the growth of H<sub>2</sub>-consuming enteropathogens. We aimed to analyze effects of single-dose lactulose ingestion on the growth of intrinsic *Escherichia coli* (*E. coli*), which can be efficiently quantified by plating and which share most metabolic requirements with *S. Typhimurium*.

### Methods

32 healthy volunteers (18 females, 14 males) were recruited. Participants were randomized for single-dose ingestion of 50 g lactulose or 50 g sucrose (controls). After ingestion, H<sub>2</sub> in expiratory air and symptoms were recorded. Stool samples were acquired at days -1, 1 and 14. We analyzed 16S microbiota composition and abundance and characteristics of *E. coli* isolates.

### Results

Lactulose ingestion resulted in diarrhea in 14/17 individuals. In 14/17 individuals, H<sub>2</sub>-levels in expiratory air increased by ≥20 ppm within 3 hours after lactulose challenge. H<sub>2</sub>-levels correlated with the number of defecations within 6 hours. *E. coli* was detectable in feces of all subjects (2 × 10<sup>2</sup>–10<sup>9</sup> CFU/g). However, the number of *E. coli* colony forming units (CFU) on selective media did not differ between any time point before or after challenge with

CRSII3\_154414/1; to WDH, partially or completely covering the salary of MK, LM, BD), the Helmut Horten Foundation (to WDH and SS), the Promedica Foundation to WDH and a grant from the Swiss National Science Foundation to BM (SNF 32473B\_156525). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

**Abbreviations:** BLAST, basic local alignment search tool; BSS, Bristol stool scale; CFU, colony forming units; CR, colonization resistance; LB, lysogeny broth; OD, optical density; PBS, phosphate buffered saline; PEG, Polyethylene glycol; SIBO, small intestinal bacterial overgrowth; *S. Typhimurium*, *Salmonella Typhimurium*.

sucrose or lactulose. The microbiota composition also remained stable upon lactulose exposure.

## Conclusion

Ingestion of a single dose of 50 g lactulose does not significantly alter *E. coli* density in stool samples of healthy volunteers. 50 g lactulose therefore seems unlikely to sufficiently alter growth conditions in the intestine for a significant predisposition to infection with H<sub>2</sub>-consuming enteropathogens such as *S. Typhimurium* ([www.clinicaltrials.gov/NCT02397512](http://www.clinicaltrials.gov/NCT02397512)).

## Introduction

Humans coexist with trillions of microbes on their body surfaces, collectively referred to as microbiota. One of the key benefits these microbes confer to their host is colonization resistance (CR), i.e. protection against invasion and infection by pathogens. It is conceivable that during gut colonization, the pathogen has to compete with the resident intestinal microbiota for nutrients and binding sites (nutrient-niche hypothesis) [1, 2]. Yet, the mechanistic details underlying the protective effect by the microbiota have not been fully elucidated.

Several perturbations can disrupt colonization resistance. Antibiotic treatment can disturb the intestinal microbiota, predisposing the host to infections with enteric pathogens including *Salmonella Typhimurium* (*S. Typhimurium*) and *Clostridium difficile* in humans [3] and in a murine model [1, 4, 5]. Likewise, according to the nutrient-niche hypothesis, providing the ecosystem with an additional nutrient should also open new niches, which might reduce colonization resistance for certain pathogens. Effects of long-term and short-term (< 1 day) perturbations might thereby differ since long-lasting interventions will trigger complex secondary responses of the gut ecosystem.

The disaccharide lactulose is used as a food ingredient (named galacto-fructose) [6, 7] and as a prescription or over-the-counter medication for constipation [8] or hepatic encephalopathy in individuals with liver cirrhosis [9]. The lactulose breath test uses lactulose to diagnose small intestinal bacterial overgrowth [10]. As lactulose cannot be absorbed during the passage through the host's small intestine [11], it reaches the large intestine where it enriches the nutrient pool available to the gut microbiota. After arrival, lactulose is fermented by certain gut microbiota members resulting in the production of short chain fatty acids (SCFA), hydrogen (H<sub>2</sub>) and methane [12, 13]. The production of these compounds explains the laxative properties and also the side effects (abdominal pain and bloating) of this drug. Based on these observations, it seems likely that the intake of lactulose might significantly alter nutrient availability in the colon and thereby change the colonic microbiota composition. Effects of short-term lactulose exposure on the gut microbiota have been insufficiently studied.

We hypothesized that lactulose may also promote gut luminal growth of Enterobacteriaceae like *S. Typhimurium* or *Escherichia coli* (*E. coli*). *E. coli* shares most metabolic requirements of *S. Typhimurium* and growth of *E. coli* in stool samples is considered a surrogate for favorable growth conditions of enteropathogens such as *S. Typhimurium* [14]. In contrast to *S. Typhimurium*, *E. coli* can grow on lactulose, i.e. by taking up and degrading this disaccharide. However, *E. coli* might also benefit directly or indirectly from the sugar monomers galactose and fructose, which are released upon degradation of lactulose by certain members of the gut microbiota [15, 16] or by fermentation products like H<sub>2</sub>. H<sub>2</sub> can serve as an electron donor for *S. Typhimurium* and other Enterobacteriaceae [17–21]. Data in our murine model of *S.*

*Typhimurium colitis* demonstrated significant attenuation of a *S. Typhimurium* mutant deficient in  $H_2$ -metabolism during initial stages of infection [18].  $H_2$  therefore constitutes a critical metabolic intermediate exploited by *S. Typhimurium* for efficient colonization of the large intestine [18].

These data from animal experiments raise the possibility that lactulose exposure might promote growth of intestinal pathogens also in humans. Since lactulose is frequently used in medical practice for instance for patients with advanced liver disease (see above), such a finding would have significant medical implications. However, the relevance of the mouse data has not been validated in humans.

Our study aims to test the hypothesis that the intake of lactulose can promote gut luminal growth of Enterobacteriaceae such as *E. coli* and *S. Typhimurium*. This effects might manifest rapidly since previous studies demonstrated alterations in microbiota composition within 24 hours after dietary interventions [22–25]. A single lactulose dose will allow monitoring direct effects of this intervention, avoiding the complexity of secondary physiological and microbiological compensatory responses upon long-term exposure.

In this study, we assessed short-term effects of lactulose intake on human volunteers using a single lactulose dose. We tested for preferential growth of Enterobacteriaceae (Enterobacteriaceae blooms) which would indicate increased risk for infection by Enterobacteriaceae in healthy individuals and by extrapolation in patients.

## Experimental procedures

### Sample size calculation

To test the primary hypothesis of our study that lactulose exposure would increase growth of *E. coli* (as a marker for Enterobacteriaceae) the following sample size calculation was performed: To be clinically relevant, the difference in *E. coli* counts after lactulose exposure compared to sucrose should be at least one order of magnitude. Bacteria concentrations were estimated to be normally distributed. In a conservative calculation, we estimated a bacteria concentration of 100'000 cfu/g with a standard deviation of 100'000 in the sucrose group and a bacteria concentration of 1'000'000 cfu/g with a standard deviation of 1'000'000 in the lactulose group. In a two-tailed unpaired analysis, a sample size of 30 individuals (15 in each group) would be required to detect this difference with a power of 90% at a significance level of 0.05 (Mann-Whitney U test).

### Study participants

For this study, 32 healthy volunteers were recruited via advertising. Exclusion criteria were a history of intestinal surgery (except hernia repair, appendectomy or anorectal surgery), pregnancy, relevant gastrointestinal symptoms (i.e., symptoms that cannot be ignored), medical or psychiatric conditions requiring ongoing management or intake of medication affecting gastrointestinal function (for instance laxatives, opioids, non-steroidal anti-inflammatory drugs, proton pump inhibitors or antibiotics within the last 4 weeks). To ensure proper handling of stool samples only employees of ETH Zurich or Zurich University, familiar with freezing and storing samples at  $-80^{\circ}\text{C}$  were recruited. All participants provided written informed consent and were compensated for their expenses. The first participant was included on the 30<sup>th</sup> of January 2015, the last participant was included into the study at the 14<sup>th</sup> of April 2015 and completed the study on 30<sup>th</sup> of April 2015. No follow up was performed (Fig 1).

Participants were screened during an interview and epidemiological data, information regarding gastrointestinal function or symptoms, medical history, medication, gastrointestinal function and life style (vegan, vegetarian or Western diet, sport [hours of exercise per week],

## CONSORT 2010 Flow Diagram

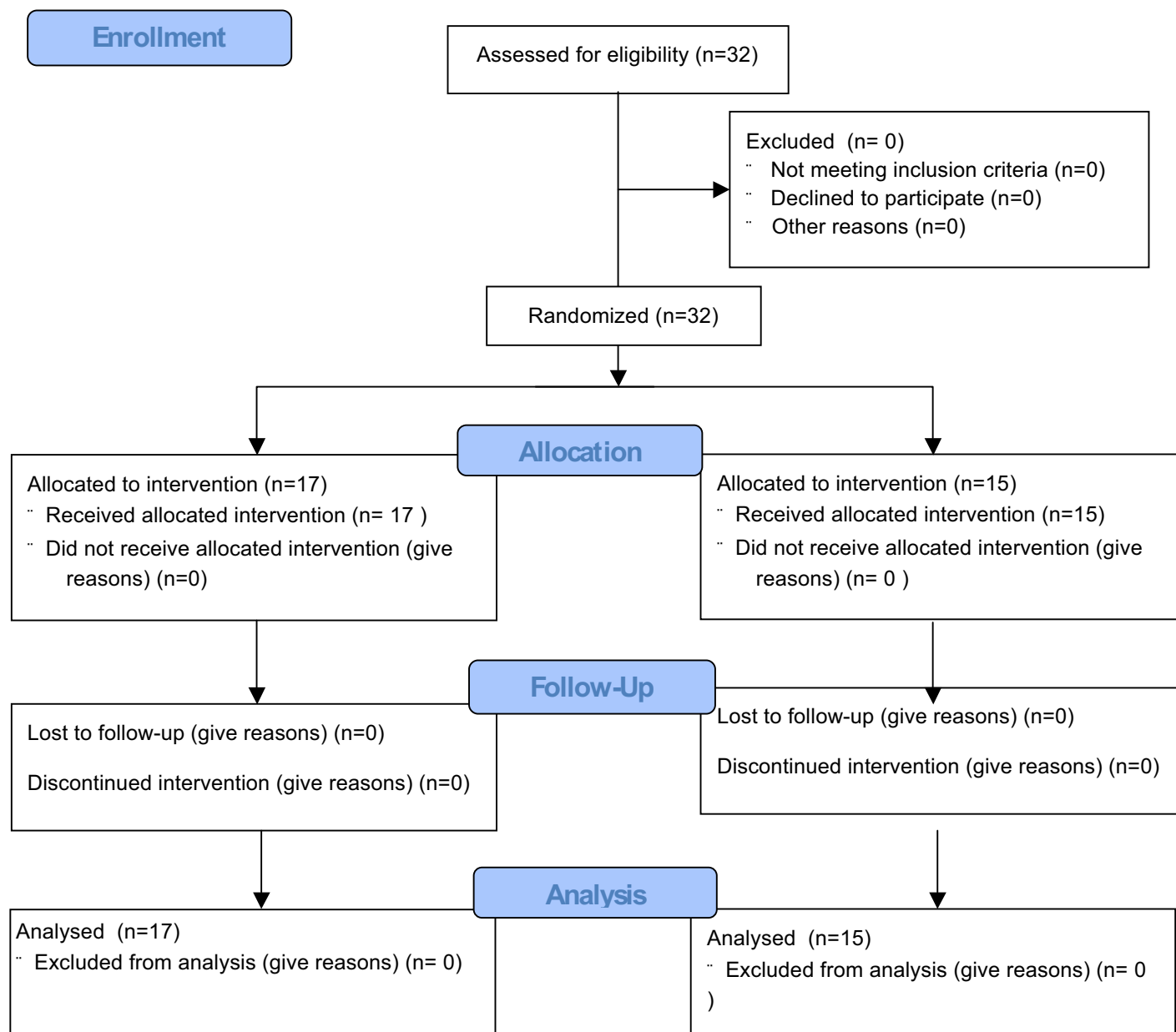


Fig 1. Consort flow chart.

<https://doi.org/10.1371/journal.pone.0206214.g001>

alcohol consumption, smoking) were recorded (Table 1). Before the intervention day, participants fasted for 12h with only water allowed prior to the intervention.

Our study was approved by the Ethics committee of Zurich county (KEK-ZH 2014–0358) and registered at clinicaltrials.gov (NCT02397512).

**Table 1. Epidemiological and clinical characteristics of the study population.** BSS: Bristol stool scale; IQR: inter-quartile range.

	Sucrose	Lactulose
<b>Number of participants</b>	15	17
<b>Age:</b> median (IQR; range)	25 (23–28.5; 21–36)	24 (22–31; 21–47)
<b>Gender:</b> female n (%)	9 (60%)	9 (53%)
<b>Stool frequency</b>		
every 2–3 days: n (%)	2 (13%)	3 (18%)
1x per day: n (%)	10 (67%)	12 (70%)
2–3x per day: n (%)	3 (20%)	2 (12%)
<b>Stool consistency (BSS)</b>		
median (IQR; range)	4 (3–4; 3–7)	4 (3–4; 3–4)
<b>Diet</b>		
Western diet	15 (100%)	15 (88%)
Vegetarian diet	-	2 (12%)
<b>Non-smokers:</b> n (%)	15 (100%)	17 (100%) <sup>a</sup>
<b>Physical exercise per week (hours)</b> median (IQR; range)	4 (3–5.5; 2–8)	3 (2–5; 0–12)
<b>Medication:</b> Yes n (%)	8 (53%) <sup>b</sup>	7 (41%) <sup>c</sup>
<b>Ethnicity</b>		
Caucasian: n (%)	14 (93%)	14 (82%)
Non-Caucasian: n (%)	1 (7%) <sup>d</sup>	3 (18%) <sup>e,f,g</sup>

<sup>a</sup> Minimal nicotine consumption in one person (3–4 cigarettes/week)

<sup>b</sup> 3x hormonal contraception, 1x thyroid hormone replacement, 3x ibuprofen

<sup>c</sup> 3x hormonal contraception, 1x ibuprofen, paracetamol, thyroid hormone replacement

<sup>d</sup> born in Vietnam, living in Switzerland for 2 years

<sup>e</sup> parents from Sri Lanka, participant born in Switzerland

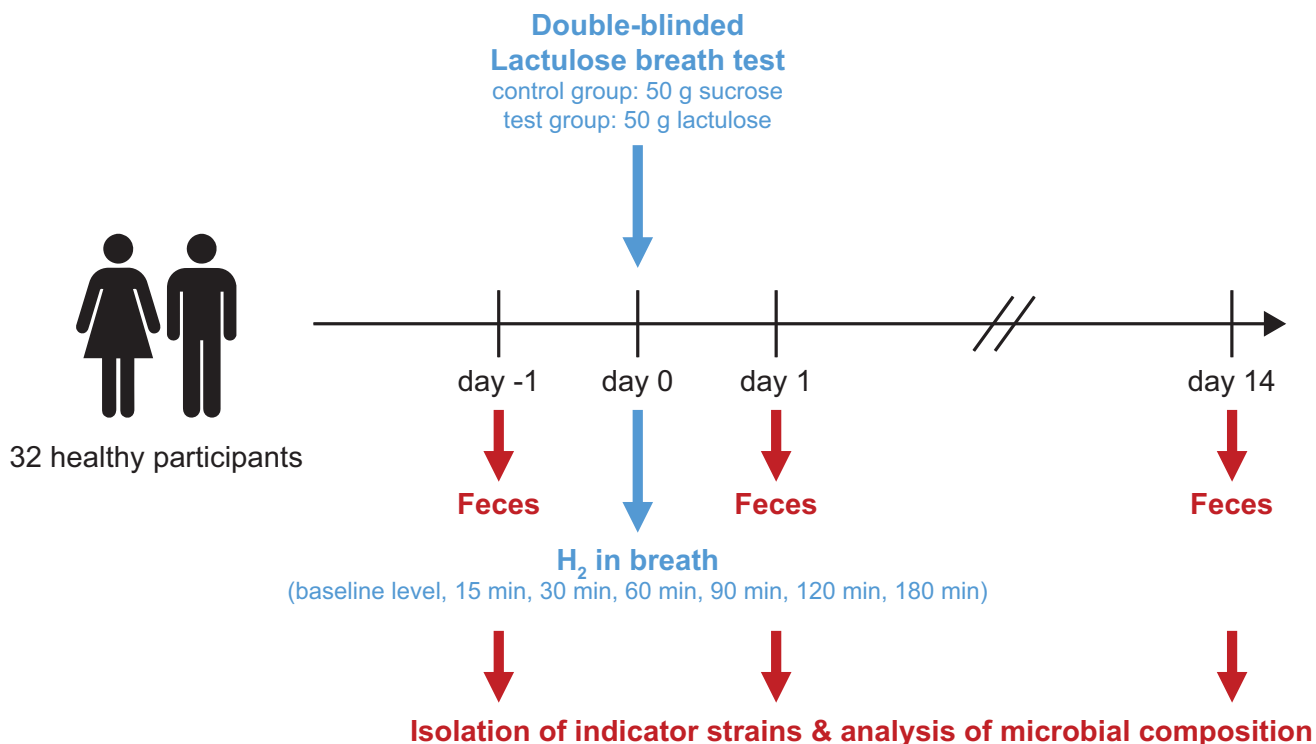
<sup>f</sup> born in Turkey, living in Switzerland for 4 years

<sup>g</sup> born in Cameroon, living in Switzerland for 3 years

<https://doi.org/10.1371/journal.pone.0206214.t001>

## Study design

The participants were randomly assigned to two groups (<http://www.graphpad.com/quickcalcs/randomize1.cfm>) using the sealed envelope method and treated in a double blinded fashion. The control group (n = 15) received a sham treatment of 50 g sucrose (Migros, Zurich) dissolved in 200 ml water. The intervention group (n = 17) consumed a drink containing 50 g lactulose (Legendall 12g, Zambon, the Netherlands) dissolved in 200 ml water (Fig 2). H<sub>2</sub> levels in parts per million (ppm) in expiratory air of participants were measured at 0 min (baseline), 15 min, 30 min, 60 min, 90 min, 120 min, and 180 min after the consumption of the respective sugar solution using the HydroCheck breath measuring device (Neomed Medizintechnik GmbH Üchtelhausen, Germany) (Fig 2). Methane content of expiratory air was recorded using Breath Collection Kit (BreathTracker SC, QuinTron USA). Due to technical issues, methane measurements could be performed only in the last 14 individuals included in our study. At all time points participants were asked to evaluate the strength of nausea, bloating, pain, stool alterations and borborygmi (bowel sounds) using a 4-point scale (1—no symptoms, 2—light symptoms which can be ignored [i.e. will not be noticed if attention is shifted to another topic], 3—marked symptoms which cannot be ignored [i.e. will typically be noticed even if individual actively shifts attention to other topics] but would not affect daily activity and 4—severe symptoms affecting daily activities. An additional questionnaire recorded bowel



**Fig 2. Overview of the design of our study.** 32 healthy participants were randomly assigned to either the control group (sucrose) or to the experimental group (lactulose). Fecal samples were collected one day before treatment, one day after treatment and two weeks after treatment for further microbial analysis. On the day of treatment, hydrogen levels were measured in expiratory air at the indicated time points.

<https://doi.org/10.1371/journal.pone.0206214.g002>

consistency (maximum Bristol stool scale, BSS) and frequency of bowel movements during 6 hours after the consumption of the respective sugar solution.

To assess effects on the resident gut microbiota, fecal samples were collected one day before, one day after, and two weeks after lactulose or sucrose exposure (Fig 2). Half of the fecal sample was stored in a test tube containing trypticase soy broth (Oxoid/ Thermo Fisher Diagnostics AG, Pratteln, Switzerland) with 15% (w/v) glycerol (Sigma-Aldrich, Buchs, Switzerland) [26]. This sample was used for the analysis of the colony forming units (CFU) per gram feces and the isolation of single bacterial strains. The other half of the fecal sample was stored in a test tube without any supplements and used for the analysis of microbiota composition. Within one hour after collection, samples were delivered by the participants and stored in a -80°C freezer provided by the investigators. To ensure proper handling of samples only individuals familiar with handling of -80°C freezers had been recruited (see inclusion criteria above) and importance of rapid sample transfer was emphasized to all individuals. Timely transfer of the fecal sample to the freezer within 30–60 min was confirmed by questionnaires to participants.

### Microbiota quantification and isolation of Enterobacteriaceae by agar plating

Fecal samples were transferred to Eppendorf tubes containing 500 µl of phosphate buffered saline (PBS) with tergitol (Sigma-Aldrich) and a sterile metal ball, weighed, and homogenized (1 min at 25 Hz, using a TissueLyser (Qiagen, Hombrechtikon, Switzerland)). Differential plating on MacConkey agar plates (Oxoid) without any antibiotics allowed identification of



bacterial population size (CFU; i.e. *E. coli*, *Salmonella* spp. and other Enterobacteriaceae) within the individual samples. In an attempt to capture the enterobacteriaceal diversity, several morphologically different colonies from each time point were picked and restreaked three times on new MacConkey agar plates. After restreaking, pure single colonies were picked and cultured in lysogeny broth (LB) for 10–12h at 37°C shaking (160 rpm). Bacterial cultures were stocked in peptone glycerol broth (peptone: Oxoid, glycerol: Sigma-Aldrich), shock frozen and stored at -80°C.

### Antibiotic susceptibility testing

Test antibiotics were chosen according to known genetic determinants of resistance [27]. Test tubes containing 2 ml LB media were inoculated with the isolated bacterial strains and incubated for 5h at 37°C shaking (160 rpm). Optical density (OD) 600 was measured and a fraction of the culture was used to inoculate new test tubes containing 2 ml LB media to obtain a final OD 600 of approximately 0.02. Liquid cultures were diluted 1:10 in sterile ddH<sub>2</sub>O and streaked with a cotton swab three times with 45° shifts on Mueller-Hinton agar to obtain a bacterial lawn of a density according to British Society for Antimicrobial Chemotherapy (BSAC) standards [28]. Antibiotic discs were placed on the agar using sterile tweezers, followed by incubation at 37°C for 18–20h. The diameter of the zone of inhibition was measured for each antibiotic and bacterial strains were classified as susceptible, intermediate, and resistant according to the Clinical and Laboratory Standards Institute (CLSI) [29].

### 16S rRNA gene analysis of isolated Enterobacteriaceae strains

For each bacterial strain, 50 µl of an overnight culture were centrifuged (5min, 8000 rpm), suspended in 100 µl sterile H<sub>2</sub>O and incubated at 99°C for 3 min. Genomic DNA was collected from supernatant after centrifugation (3min, 8000 rpm). 8 µl DNA was amplified by PCR in a 100 µl reaction mixture containing 10 µl buffer (5 PRIME PCR master kit), 5 U Taq polymerase (5 PRIME PCR master kit), 8 µl 2.5 mM dNTPs, 53 µl ddH<sub>2</sub>O and 10 µl of each forward primer (fD1: 5' -AGAGTTTGATCCTGGCTCAG-3') [30] and reverse primer (rP1: 5' -ACGGTTACCTTGTTAGCACTT-3') [31] diluted 1:10 in ddH<sub>2</sub>O (final primer concentration 1 µM). The amplification program consisted of an initial denaturation step of 5 min at 94°C; followed by 35 cycles of 1 min at 94°C, 1 min at 43°C, and 2 min at 72°C; and a final extension of 7 min at 72°C. A negative control was included in each batch of PCR reactions. Correct PCR product size was confirmed using agarose gel electrophoresis and ethidium bromide staining, DNA fragments were excised under UV light and extracted using the Wizard SV Gel and PCR Clean-Up System (Promega, Dübendorf, Switzerland).

Excised DNA fragments were sent for sequencing at Microsynth AG Balgach, Switzerland. The sequences were annotated by the basic local alignment search tool (BLAST) [32] for taxonomical identification. Entries were sorted by "Max score" and the top record used to annotate the genus and, if reliable, also the species classification for each bacterial strain. The sequence alignment tool MUSCLE [33] and SplitsTree 4.13 [34] were used to construct phylogenetic trees based on the BLAST annotations.

### DNA extraction for 16S gene-based microbiota composition analysis

DNA was extracted using the QIAamp Stool Mini Kit (Qiagen) with the following changes in the disruption and homogenization steps: 700µl of Buffer ASL, 2 spoons of small glass beads (0.1mm; Bio Spec products) and 1 spoon of large glass beads (0.5–0.75mm; Schieritz&Hauenstein, Laufen, Switzerland) were added to each stool sample. The samples were mixed using the TissueLyser (Qiagen) (3 min, 30Hz) and heated for 5 min at 95°C. Next, samples were

again mixed using the TissueLyser (3 min, 30Hz), centrifuged at full speed for 1 min and the supernatants were transferred to a new 2 ml microcentrifuge tube. The pellet was suspended in 200  $\mu$ l of Lysis Buffer (20mg/ml lysozyme (Sigma-Aldrich); 20mM Tris•HCl, pH 8.0; 2mM EDTA; 1.2% Triton) and incubated at 37°C for 30 min. Afterwards, 500  $\mu$ l of Buffer ASL were added to each sample followed by mixing using the TissueLyser (3min, 30Hz) and heating the suspension for 5 min at 95°C. All samples were mixed again using the TissueLyser (3 min, 30Hz) and centrifuged at full speed for 1 min. The supernatants were transferred to the same 2 ml tube than used before. Half of an InhibitEX tablet (provided in Kit) was added to each sample and tubes were vortexed immediately until the tablet was completely suspended. Next, samples were incubated for 1 min at room temperature, centrifuged at full speed for 3 min and supernatants were transferred into a new 1.5 ml microcentrifuge tube. The samples were again centrifuged at full speed for 3 min. Meanwhile, 35  $\mu$ l of proteinase K (provided in Kit) was added to a new 2 ml tube and supernatants from the previous centrifugation step were transferred to the 2 ml tube containing the proteinase K. 200  $\mu$ l of Buffer AL were added and samples were vortexed for 15 sec and incubated at 70°C for 10 min. The next steps were performed according to the manual instructions. The final elution step was performed using 100  $\mu$ l Buffer AE (pre-heated to 70°C) added to the membrane, then samples were incubated for 1 min at room temperature and centrifuged at full speed for 1 min to elute DNA.

### Library preparation and sequencing for microbiota composition analysis

The 16S rRNA gene libraries were produced using the NEXTflex® 16S V4 Amplicon-Seq Kit 2.0 (Barcodes 1–96; Bioo Scientific, Austin, Texas, USA). The input concentration of genomic DNA was adjusted to 30 ng/ $\mu$ l for each PCR reaction. The library preparation was performed following the manufacturer's instructions with the differences that the reaction volume was reduced to 25  $\mu$ l and a modified primer pair was used for the first PCR reaction (final primer concentrations 0.5  $\mu$ M). Instead of the original primer pair 515f-806r, the degenerative primer 515F (5'–GTG**Y**CAGCMGCCGCGTAA–3') and 806rB (5'–GGACTAC**N**VGGGTWTCTAAT–3'), as described in [35, 36] were used. The first PCR reaction was performed using Q5 High-Fidelity DNA Polymerase (BioConcept, NEB, Allschwil, Switzerland) under the following cycling conditions: 1) initial denaturation: 95°C for 4 min; 2) denaturation: 95°C for 30 sec; 3) annealing: 56°C for 30 sec; 4) extension: 72°C for 90 sec; 5) final extension: 72°C for 4 min. Cycles 2–4 were repeated 8 times. A negative control was included in each batch of PCR reactions, which showed no signs of amplification. After each reaction, the PCR products were cleaned up using AMPure XP magnetic beads (Beckman Coulter SA, Nyon, Switzerland). The clean PCR products were eluted in 20  $\mu$ l of resuspension buffer and used in a second PCR reaction for producing multiplexed samples, respectively. The quantity of the amplicons was measured by a Qubit 3.0 fluorometer (Thermo Fisher Scientific). The quality of the amplicons was evaluated using a Fragment Analyzer (Advanced Analytical, Ankeny, IA, USA). The length of the barcoded amplicons was approximately 450 bp. The amplicons were pooled at equimolar concentrations and diluted to a final concentration of 60 ng DNA per 20  $\mu$ l before loading on the Illumina MiSeq platform. Sequencing was performed at 2 x 250 bp read length at the Functional Genomics Center Zurich. All sequences obtained during this project have been deposited at the European Nucleotide Archive under the accession number: PRJEB28417

### Microbiota composition analysis

Initial analyses were performed using USEARCH (version 9.1.13) [37] using custom scripts that performed the following steps: paired reads were merged and quality-filtered using the *fastq\_mergepairs* command with default settings. Merged reads were filtered using the

*fastq\_filter* command (-fastq\_maxee 1.0) and only merged reads with perfect primer matches and a minimum length of 100 bp were selected. Sequences were de-replicated using the *fastx\_uniques* and clustered into operational taxonomic units (OTUs) at 97% with chimera removal using the *cluster\_otus* command (-minsize 2). OTU abundances for each sample were quantified using the *usearch\_global* command (-strand both; -id 0.97). Taxonomic annotation was performed by matching OTU sequences against the SILVA database (version 128) using the *usearch\_global* command (-id 0.90; -maxaccepts 20; -maxrejects 500; -strand both; -top\_hits\_only).

Further analyses were performed in RStudio (version 1.0.143 based on [R] version 3.3.3) using the libraries *vegan* [38] and *ggplot2* [39]. Read counts were rarefied using the function *rrarefy* to 19'000 reads per sample based on the sample with the lowest number of sequencing reads after excluding five samples that had too few reads. Shannon's diversity index for each sample was calculated using the *diversity* function. For performing a Principal Coordinate Analysis and for calculating intra-individual dissimilarity (grouped by time point or individual), Bray-Curtis distances were computed between pairs of samples using the *vegdist* function.

## Statistical analysis

Patient data with time curves for symptoms after lactulose/ sucrose exposure were analyzed using two-way repeated measures ANOVA (GraphPad Prism Version 7.02 La Jolla, CA, USA, [www.graphpad.com](http://www.graphpad.com)). Correlation analysis for symptoms, hydrogen levels and Enterobacteriaceae counts was performed using Spearman R correlation of GraphPad Prism. Group comparisons for Enterobacteriaceae counts was done using the Mann-Whitney U test of GraphPad Prism. The sample size calculation was performed using G\*Power Version 3.1.9.2 [40]. A p-value of less than 0.05 was considered significant.

Microbiota composition analysis was done using USEARCH (version 9.1.13) [37] and R Studio (version 1.0.143 based on [R] version 3.3.3) with the libraries *vegan* [38] and *ggplot2* [39] as described above. Significant differences between time points for each treatment group as well as phylum-level taxonomic abundances were calculated by paired Wilcoxon tests with FDR correction (0.05) for multiple testing. We also determined intra-individual dissimilarity using the Bray-Curtis distance and compared different sampling time points by paired Wilcoxon tests. For this analysis only individuals with samples from all three time points were included (n = 14 for sucrose, n = 13 for lactulose). To assess sources of variation in our data set, we tested the relative contribution of individuality and treatment to the overall variation of microbial compositions by permutational MANOVA (Permutations = 999).

## Results

### Study population

Our study recruited 32 healthy volunteers (Fig 1), 18 out of 32 (56%) female of young age (median 25, IQR: 22.75–30, Table 1). All individuals had normal bowel habits (frequency of bowel movements every 2–3 days to 2–3 times per day, median BSS 4), were non-smokers and most individuals followed a Western diet with regular physical activity. Medical history did not reveal relevant medical conditions or gastrointestinal complaints; medication included only analgetics, thyroid hormone replacements and hormonal contraception (Table 1). Thus, we recruited a homogenous healthy study population.

## Symptoms upon sucrose and lactulose ingestion

Individuals were randomized with 17 individuals in the intervention group with lactulose ingestion and 15 individuals in the control group with sucrose ingestion, respectively (Fig 2). Following ingestion of the respective disaccharide, 14/17 (82%) of individuals in the lactulose group but only 3/15 (20%) of individuals in the control group reported physical symptoms. Within three hour of observation on a five-point scale, scores for bloating, diarrhea, borborygmi (bowel sounds) and abdominal pain of the lactulose group were significantly higher compared to the sucrose group (bloating: median 2 [range 1–4] vs. 1 [1–2],  $p = 0.0006$ ; diarrhea: median 1 [range 1–5] vs. 1 [1–1],  $p = 0.0128$ ; borborygmi: median 2 [range 1–4] vs. 1 [1–2],  $p = 0.0005$ ; abdominal pain: median 1 [range 1–3] vs. 1 [1–2],  $p = 0.0163$ ; Fig 3, S1 Table).

On separate questioning, 13 out of 17 individuals reported diarrhea (defined as  $\geq 3$  bowel movements within 6 hours after lactulose intake); diarrhea was accompanied by loose stool consistency (Bristol stool scale, BSS  $\geq 5$  in all individuals with diarrhea). 5 of those reported  $\geq 4$  bowel movements with BSS 7. The remaining 4 out of 17 individuals did not respond to lactulose treatment with no defecation during 6 hours in 3 participants and 1 defecation BSS 5 in the remaining participant. No diarrhea was observed in the control (sucrose).

After lactulose exposure,  $H_2$  levels in expiratory air gradually increased for the next 90 min and remained at significantly higher level with lactulose compared to sucrose ( $p < 0.001$ ) until the end of the observation period (180 min, Fig 4).  $H_2$  levels varied widely (0–146 ppm); for three individuals of the lactulose intervention group,  $H_2$  levels in expiratory air never increased for more than 20 ppm over baseline; these individuals are by definition  $H_2$  non-producers [10].

For 14 out of 32 individuals (5 in the sucrose group, 9 in the lactulose group) methane ( $CH_4$ ) measurements were also taken (Fig 4B); for technical reasons, only the last 14 individuals included into our study could be tested. However, only a single individual had increased  $CH_4$  levels at baseline (15 ppm) and for no individual  $CH_4$  levels exceeded 10 ppm over baseline. For the three  $H_2$  non-producers no  $CH_4$  measurements were available.

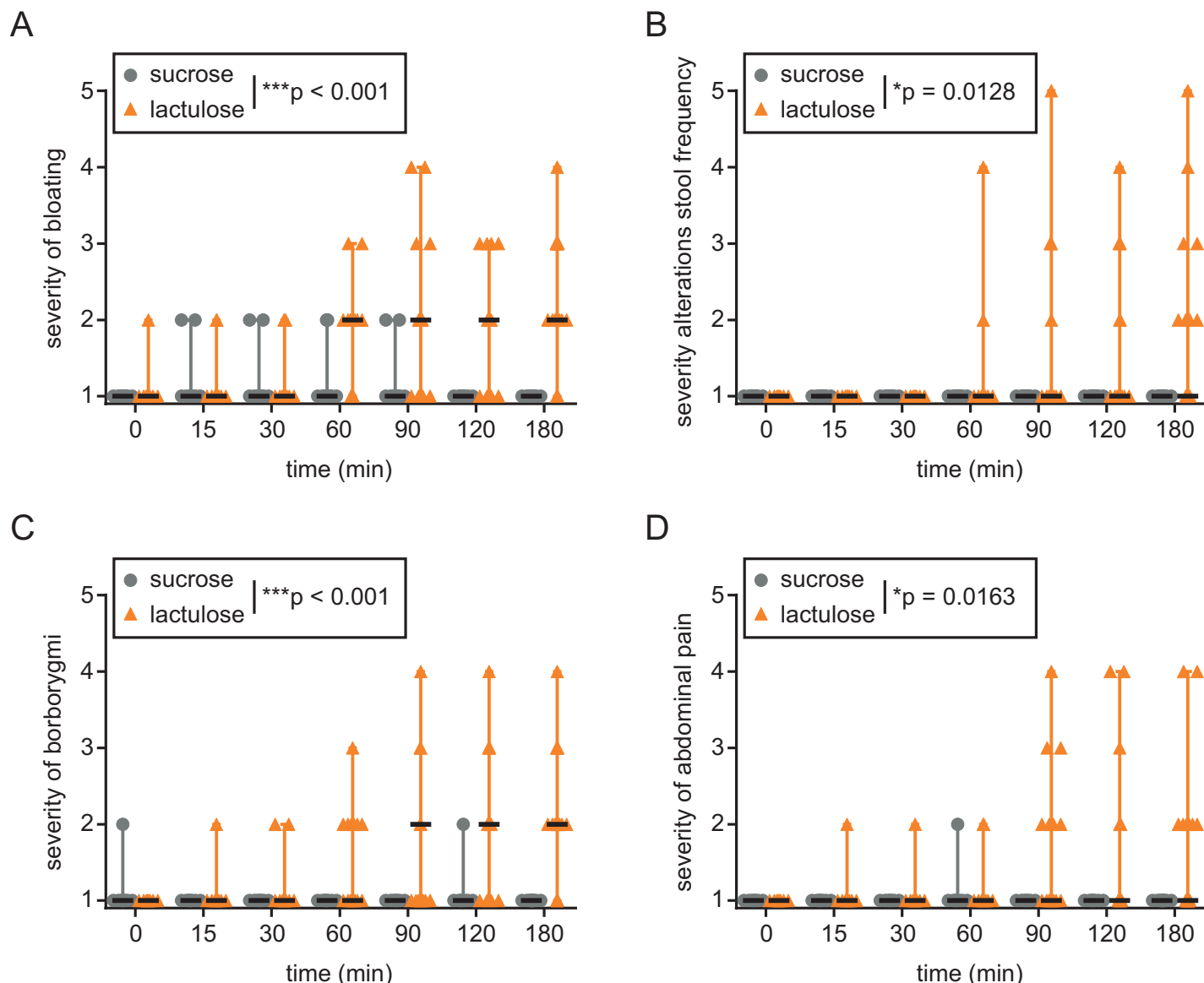
$H_2$  levels in expiratory air correlated with number of defecations ( $p = 0.024$ , Fig 4C); correlation analysis with stool consistency (BSS) revealed a non-significant trend ( $p = 0.052$ , Fig 4D), suggesting that the intestinal response to lactulose was associated with  $H_2$  production.

## Bacterial growth in stool samples of lactulose and sucrose treated subjects

We tested whether lactulose ingestion and exposure of the intestine to excess  $H_2$  increases levels of *E. coli* and related Enterobacteriaceae in stool samples. Consistency of collected stool samples (as judged by in inspection of collected material) was similar at all time points (day -1, day 1 and day 14), arguing against significant confounding by sample dilution. Stool samples were plated on selective media to quantify *E. coli* one day before (baseline), one day after, and two weeks after disaccharide exposure (Fig 5). At all time points, no difference in bacterial numbers could be detected (Fig 5A–5C). Variation within the two groups was high, with bacterial densities ranging from  $3 \times 10^2$  to  $10^9$  colony forming units (CFU) per g fecal sample in the control group and  $2 \times 10^2$  to  $8 \times 10^8$  CFU per g in the lactulose group (Fig 5A–5C).

CFU counts remained stable over time and no difference for time points could be detected in a paired analysis ( $p > 0.05$  for all comparisons, Wilcoxon matched pairs signed-rank test). We found significant correlations of the number of CFUs at baseline, day 1 and day 14 in the group with lactulose treatment (Fig 5D–5F), indicating stability of *E. coli* population size over time and upon lactulose perturbation.

Considering the  $H_2$  metabolism in *E. coli* and other organisms (e.g. *S. Typhimurium*, *Yersinia*, *Campylobacter*, *Shigella* [17–21]) our data argue against a pronounced effect of increased

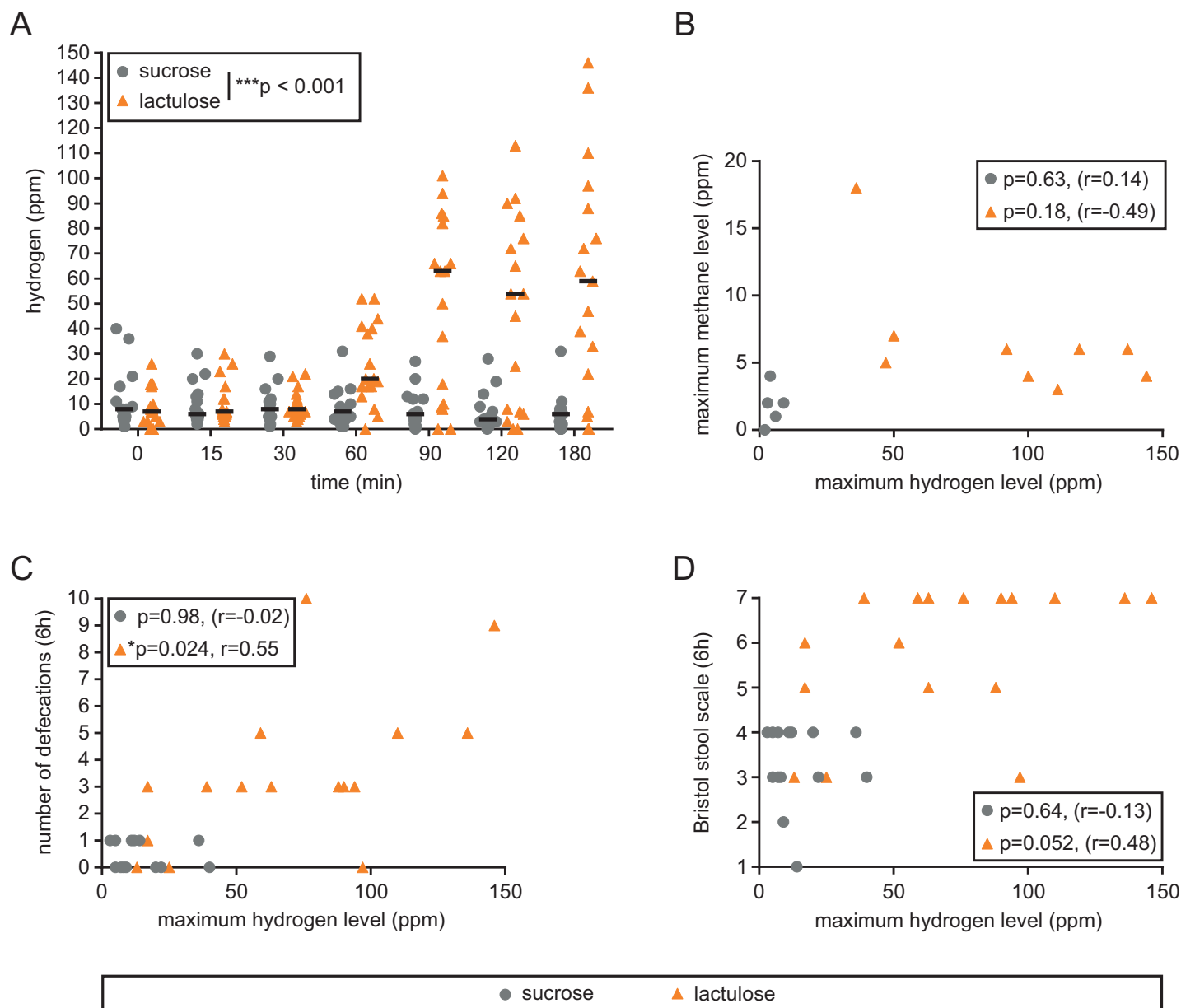


**Fig 3. Symptoms upon lactulose or sucrose ingestion.** Severity of (A) bloating, (B) stool alterations, (C) borborygmi and (D) abdominal pain was rated by individual participants according to the following scale: 1—no symptoms, 2—symptoms which can be ignored, 3—symptoms which cannot be ignored, but don't affect daily activities, 4—symptoms affecting daily activities, 5—symptoms dominating and severely inhibiting daily activities.  $*p < 0.05$ ,  $***p < 0.001$ ; Two-way repeated measures ANOVA.

<https://doi.org/10.1371/journal.pone.0206214.g003>

H<sub>2</sub> levels on the growth of potential invading enteropathogens. In line with this interpretation, the number of CFU at the day after lactulose exposure (or the ratio of CFU between time points) did not correlate with maximum H<sub>2</sub> levels in expiratory air at the intervention day (Figure A in [S1 Fig](#)).

Number of CFUs or ratios of CFUs between days also did not correlate with number of defecations after lactulose exposure (Figure B in [S1 Fig](#)). We noted a positive correlation between CFU and stool consistency (BSS) and the ratio of CFUs on day -1: day 1 in the control group but not in the lactulose group (Figure C in [S1 Fig](#)).



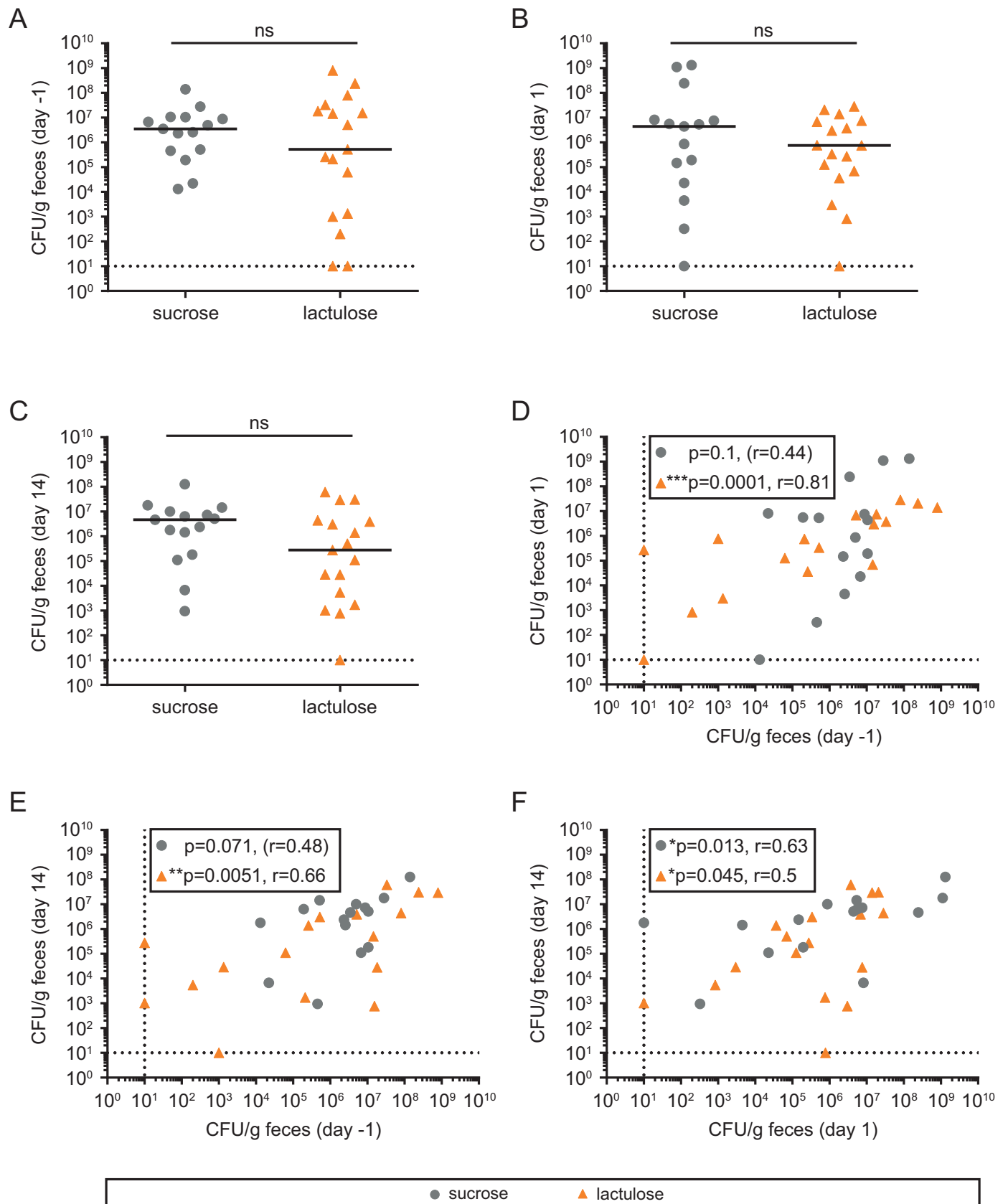
**Fig 4. Hydrogen and methane levels and symptoms following lactulose ingestion.** (A) Hydrogen levels in parts per million (ppm) following lactulose or sucrose (control) ingestion. \*\*\* $p < 0.001$ ; two-way repeated measures ANOVA. (B) Correlation between maximum (max) hydrogen and methane levels. (C) Correlation between maximum hydrogen levels and number of defecations during 6 hours following lactulose or sucrose ingestion. (D) Correlation between maximum hydrogen levels (max) and stool consistency (Bristol stool scale) following lactulose or sucrose ingestion. r: Spearman R correlation coefficient.

<https://doi.org/10.1371/journal.pone.0206214.g004>

No *Salmonella enterica* colonies were detected in any of the stool samples, as judged from the absence of color-less colonies on the MacConkey agar plates and 16S sequencing in ambiguous cases.

### Phylogenetic analysis of stool isolates

To confirm species affiliation of isolated Enterobacteriaceae, bacterial strains from 30 participants at one day after treatment were analyzed by sequencing the 16S rRNA gene. Sequences





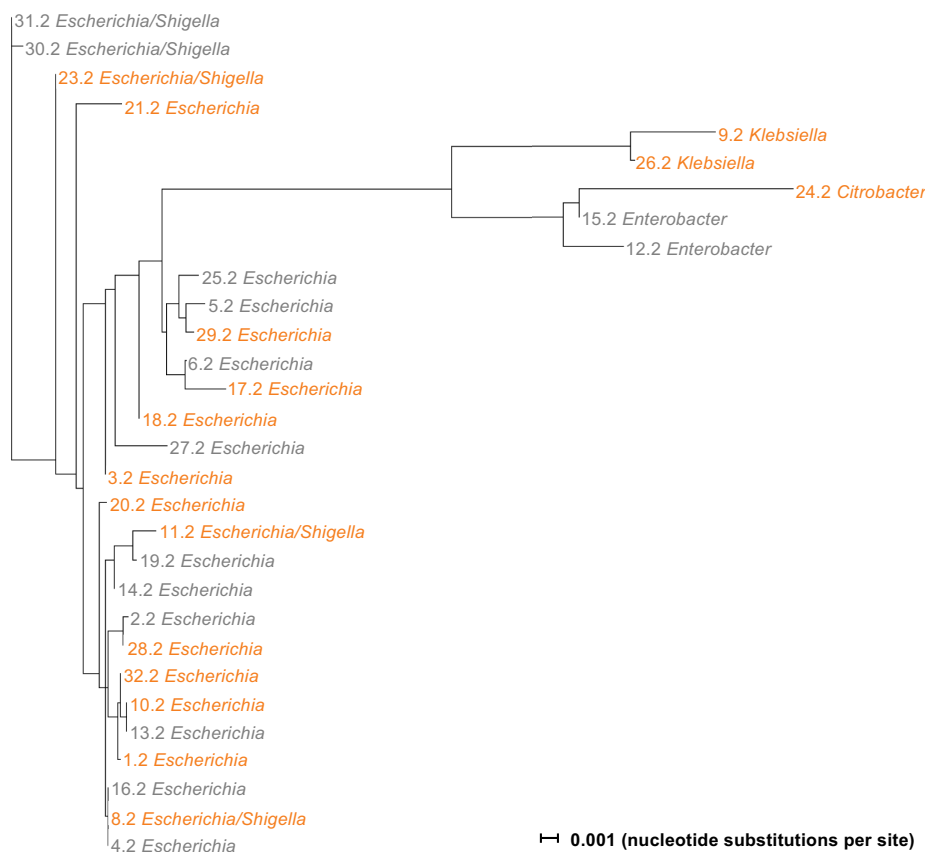
**Fig 5. *E. coli* counts in stool samples at different time points.** (A-C) Stool samples were plated on selective agar without antibiotics to quantify *E. coli* counts (CFU). ns = not significant; Mann-Whitney U test. (D-F) Correlations of *E. coli* counts within the feces collected at different time points. ns: not significant; \* $p < 0.05$  = significant; \*\* $p < 0.01$ ; \*\*\*\* $p < 0.001$ ; r: Spearman R correlation coefficient. Dashed lines = detection limit.

<https://doi.org/10.1371/journal.pone.0206214.g005>

were classified using the basic local alignment search tool (BLAST). All bacteria isolated from MacConkey agar were found to belong to the family of Enterobacteriaceae with a large cluster of 20 *E. coli* related species (20 *E. coli*, 5 *E. coli*/ *Shigella*) and a distantly related cluster of 2 *Klebsiella*, 2 *Enterobacter* and 1 *Citrobacter* species (Fig 6).

## Antibiotic resistance

Annotated human bacterial isolates were tested for resistance against different antibiotics. Seven antibiotics (imipenem, cefepime, gentamicin, kanamycin, ciprofloxacin, and trimethoprim/ sulfamethoxazole) from 5 classes (carbapenems, cephalosporins, aminoglycosides, fluoroquinolone and folic acid synthesis inhibitors) were chosen according to known genetic determinants of resistance in Enterobacteriaceae to evaluate resistance status in environmental settings [27].



**Fig 6. Phylogenetic tree of Enterobacteriaceae strains isolated one day after treatment.** Tree showing the relatedness between bacterial strains ( $n = 30$ ) isolated from participant samples one day after treatment with sucrose (grey) or lactulose (orange). 16S rRNA sequences were annotated by BLAST and aligned using MUSCLE and SplitsTree. *Escherichia/Shigella* thereby indicates a strain with a sequence compatible with both, *E. coli* and *Shigella*. Horizontal lines indicate nucleotide substitutions per site. Please note that *E. coli* and *Shigella* are extremely closely related. Thus, the detection of *Escherichia/Shigella*-like sequences does not imply that *Shigella* spp. were present in the stools of any of the participants.

<https://doi.org/10.1371/journal.pone.0206214.g006>



All bacterial strains tested were susceptible to imipenem, cefepime, and kanamycin (S2 Table). Bacterial resistance against ampicillin was common (11 out of 30 strains, 37%) followed by sulfamethoxazole/trimethoprim (7 out of 30 strains, 23%), ciprofloxacin (2 out of 30 strains, 7%) and gentamicin (1 out of 30 strains, 3%). Five bacterial strains were resistant to two or more antibiotics (S2 Table). No extended spectrum beta-lactamase producing strains were identified.

### Stability of microbiota upon lactulose exposure

We used 16S rRNA gene sequencing to analyze the taxonomic composition of the intestinal microbiota after lactulose challenge. Overall, we observed no significant difference in bacterial diversity between lactulose and sucrose treated individuals at day 1 and day 14 compared to pre-treatment conditions (Fig 7A). The microbiota composition was also highly similar between lactulose and sucrose treatment. An example for phylum level compositions is provided in Fig 7B. The mean relative abundance of Enterobacteriaceae was 0.4% (interquartile range 0.07% - 0.6%) of all intestinal bacteria detected by 16S sequencing. No significant alteration in the relative abundance of the family Enterobacteriaceae and genus *Escherichia-Shigella* (represented by a single OTU with a 100% sequence match to *E. coli*) was detected neither within nor between treatment groups (false discovery rate (FDR)-corrected Wilcoxon tests) and sampling time points within treatments (paired FDR-corrected Wilcoxon tests, S2 Fig).

We observed intra-individual shifts in the dissimilarity of microbial composition between the different sampling time points (days -1, 1, 14). Nevertheless, the shifts were similar in size for lactulose and sucrose treated individuals. Furthermore, we did not observe any clustering of samples by treatment (principal coordinate (PCo); analysis Fig 7C). Similarly, we detected no significant intra-individual dissimilarity changes before and after lactulose/ sucrose ingestion (Fig 7D). Additionally, there was no correlation between intra-individual dissimilarity (i.e. dissimilarity between time points) and BSS or H<sub>2</sub> levels (Fig 7E).

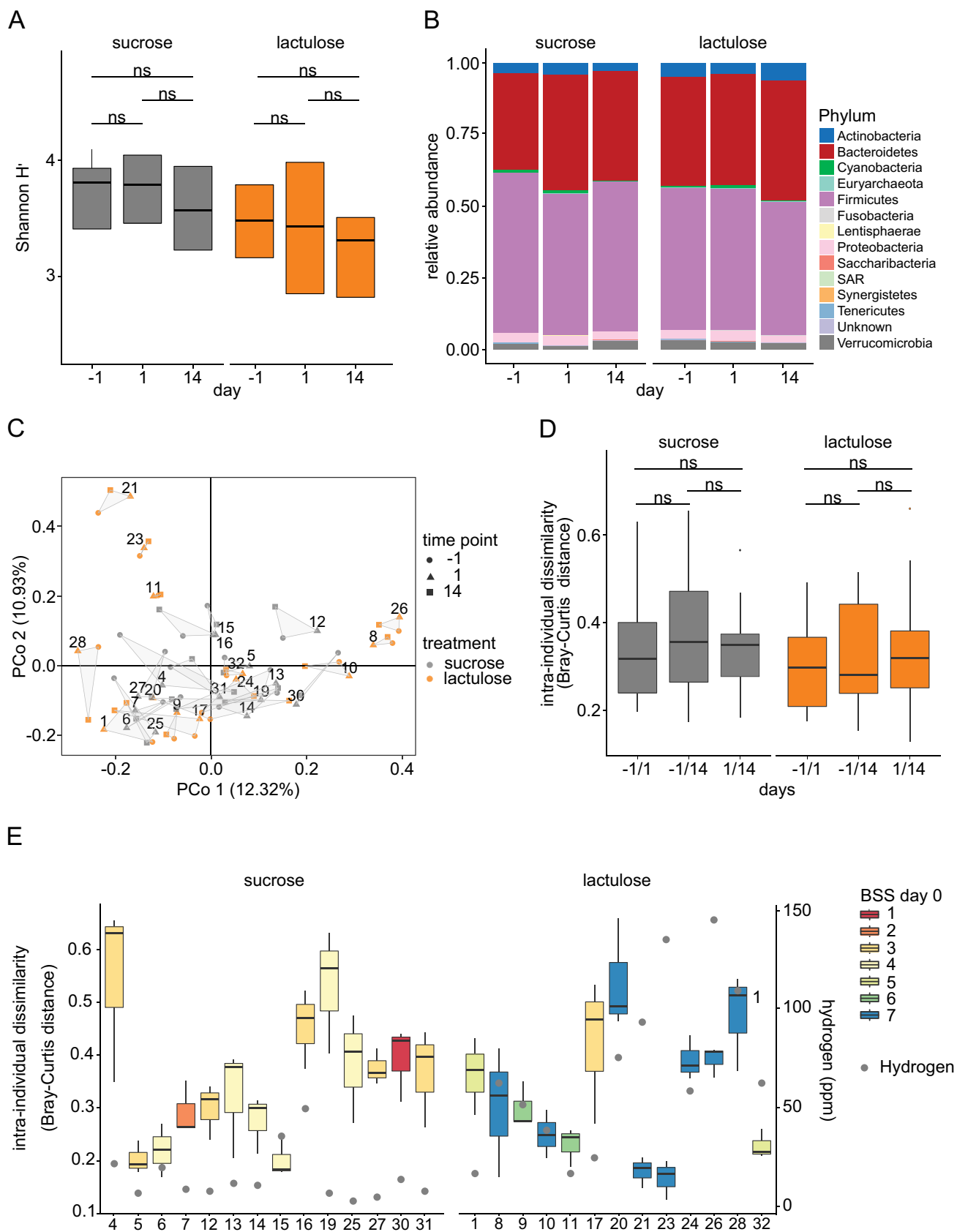
Given the absence of a strong treatment-related effect on microbial composition, we sought to assess the sources of variation in our data set. To this end, we tested the relative contribution of individuality and treatment to the overall variation of microbial compositions (permutational MANOVA). For both treatments, we found individual variations but not treatment to explain the vast majority of the variation (effect of individuals: sucrose group—R<sup>2</sup>: 0.77,  $p = 0.001$ ; lactulose group—R<sup>2</sup>: 0.82,  $p = 0.001$ ; effect of treatment: sucrose group—R<sup>2</sup>: 0.01 n.s.; lactulose group—R<sup>2</sup>: 0.007, n.s.).

### Discussion

We performed a placebo controlled randomized controlled study to test effects of a single dose lactulose challenge on gastrointestinal microbiota composition and wellbeing in a homogeneous group of young healthy individuals. The following key observations were made: i) Lactulose caused an increase in H<sub>2</sub> levels in expiratory air and diarrhea in the vast majority of individuals in the lactulose group. ii) In the lactulose group, H<sub>2</sub> levels correlated with severity of diarrhea (i.e. number of defecations). iii) Lactulose exposure did not increase the number of CFU of *E. coli* in stool samples collected 1 day after exposure. iv) The microbiota composition at that time point was also not significantly affected by single-dose lactulose challenge compared to the sucrose control, indicating that individual day-to-day variations of the microbiota are much stronger than the perturbation by a single 50 g lactulose ingestion.

### Effectiveness of a single dose lactulose as a laxative

Usage of lactulose for the treatment of acute or chronic constipation is well established [41]. Recent randomized controlled trials used lactulose as a standard treatment for comparative



**Fig 7. A single dose of lactulose (50 g) has no significant effect on fecal bacterial community structure a day or two weeks after treatment.** (A) Shannon diversity index shows no significant change (paired Wilcoxon test,  $P > 0.05$ ) one day or two weeks after treatment (sucrose  $n = 15$ , lactulose  $n = 17$ ) for each group. (B) Relative phylum abundance before (day -1) and after (day 1 and day 14) ingestion of sucrose ( $n = 15$ ) or lactulose ( $n = 17$ ). Colors indicate bacterial phyla, none on which were significantly different between time points. (C) Principal coordinate (PCo) analysis of microbiota composition with symbols and colors denoting sampling time point and treatment (sucrose, lactulose), respectively. The numbers denote the different individuals with samples from all three time points. (D) Intra-individual dissimilarity (Bray-Curtis) of microbial composition in samples collected at different sampling time points (day—1 to 1, day -1 to 14 and day 1 to 14) and treatments with sucrose ( $n = 14$ ) and lactulose ( $n = 13$ ). Only individuals with samples from all three time points with a rarefaction cut-off of  $> 19'000$  were included in the intra-individual dissimilarity analysis (Bray-Curtis) of the microbiota composition. No significant shifts in bacterial composition were detected between different time points (paired Wilcoxon test,  $p > 0.05$ ). (E) Intra-individual dissimilarity (Bray-Curtis) of fecal microbial composition between the three sampling time points. The color key indicates the Bristol stool scale (BSS) of the same day (i.e., day 0) and after the lactulose/ sucrose challenge. The grey dots show maximal hydrogen levels in parts per million (ppm) following lactulose or sucrose ingestion.

<https://doi.org/10.1371/journal.pone.0206214.g007>

testing against sodium polyethylene glycol (PEG) [42, 43], sodium picosulfate [42] and Chinese or Pakistan herbal medicine [44, 45]. Effects of PEG were stronger than lactulose in a Cochrane meta-analysis [46] and PEG or liquid paraffin were more effective in children [47] even though these differences are small and possibly not clinically relevant. Cost-effectiveness of PEG and lactulose was virtually identical [48]. Interestingly, lactitol might be similarly effective, but more palatable with lesser side effects than lactulose [49].

We are not aware of a recent placebo-controlled randomized controlled trial for one-time application of lactulose in young healthy individuals. Thus, our study provides valuable information on the effectiveness of lactulose in healthy individuals. We observed diarrhea (i.e.  $\geq 3$  bowel movements in 6 hours) in 14 out of 17 (82%) of healthy participants ingesting lactulose which was accompanied by at least moderate borborygmi, bloating, flatulence (14 out of 17, 82% for each symptom) and abdominal pain (8 out of 17, 47%).

### Methane levels in expiratory air and H<sub>2</sub> non-producers

A fraction of patients and healthy individuals contain a methanogenic organism such as *Methanobrevibacter smithii* [50]. Hydrogenotrophic methanogens use 4 molecules of H<sub>2</sub> and 1 molecule of carbon dioxide (CO<sub>2</sub>) to produce 1 molecule of methane (CH<sub>4</sub>) [51]. Due to this additional metabolization step, the CH<sub>4</sub> peak will be delayed and due to the 4:1 ratio of H<sub>2</sub> and CH<sub>4</sub>, the methane peak will be at a lower level compared to H<sub>2</sub>. Therefore, a cut-off of 10 ppm for CH<sub>4</sub> is suggested [10]. Furthermore, individuals with a methanogenic microbiota usually have increased levels of CH<sub>4</sub> even at baseline, but fail to produce measurable H<sub>2</sub> upon appropriate testing [10]. In our analysis, methane measurements from only 14 individuals were available and only for one person, methane levels exceeded the threshold of 10 ppm. Our study included three H<sub>2</sub> non-producers (18%) without any increase in H<sub>2</sub> levels, well within the expected range of 2–43% in healthy individuals or patients [52]. However, no methane measurements for these individuals are available.

### Correlation of symptoms with H<sub>2</sub> levels

The healthy human intestine contains 30–200 ml (average 100 ml) gas, which mainly consists of H<sub>2</sub>, CO<sub>2</sub> and CH<sub>4</sub> [10, 53]. Both, H<sub>2</sub> and CH<sub>4</sub> are exclusively produced by the bacterial microbiota [50].

Our study found a significant association of peak H<sub>2</sub>-levels with severity of diarrhea upon lactulose challenge in healthy individuals, arguing for a relationship between H<sub>2</sub>-production and symptoms after lactulose challenge. The association of results of the lactulose breath test with a diagnosis of irritable bowel syndrome (IBS) or IBS symptoms is controversial. A large analysis failed to find differences in IBS patients vs. healthy controls [54]. However, the H<sub>2</sub> response in the lactulose breath test was related to bloating in IBS patients [55–57]. Similarly, a

recent study using a large cohort of Chinese IBS patients found both, high H<sub>2</sub> levels (area under the curve) in breath tests and visceral hypersensitivity to be risk factors for bloating and borborygmi and overall symptom burden [58].

Methane data were available only for a subset of participants and no correlation with symptoms could be established. Previous studies confirmed an association of methane levels in expiratory air with constipation [59, 60] and the severity of constipation correlated with methane levels [59].

### Stability of the microbiota upon single-dose lactulose challenge

In our study, no significant effects of a single dose of 50 g lactulose on microbiota composition could be detected after 1 day or after 14 days (S2 Fig). The number of *E. coli* counts in our study ( $10^4$  to  $10^8$  cfu/g feces, Fig 5) and the mean relative abundance of Enterobacteriaceae (0.4% of all intestinal bacteria detected by 16S sequencing) are well in line with numbers described in the literature [61–65], indicating a normal intestinal microbiota before and after lactulose challenge. Previous diet intervention studies showed that short-term macronutrient shifts reversibly alter gut microbiota composition within 24 hours even though broad patterns of microbiota composition similarities (enterotypes) remain stable [22–25]. Our PCo analysis showed that lactulose challenge did not significantly alter the microbial composition since similar patterns of small-scale shifting were observed for lactulose and sucrose, alike (day 1 and day 14). Instead, our data argue for a stability of microbiota compositions with daily variations dominating over changes due to a single intervention with 50 g lactulose, even though diarrhea was observed in the majority of individuals. However, the data of this study provides no information about short-term microbiota compositional changes immediately after lactulose challenge, nor about site-specific effects within the small intestine or the right-sided colon.

### Effects of lactulose on the risks for intestinal infections

Prebiotics are food ingredients, which are neither digested nor degraded in the stomach or small intestine, but fermented by the gut microbiota, leading to a selective stimulation of the growth of certain intestinal bacteria and may thereby reap potential benefits for the host [66]. Lactulose, along with inulin and other oligosaccharides are considered prebiotics and *Lactobacillus* and *Bifidobacterium* are considered significant target genera for the associated prebiotic effects [66, 67].

Since over 50 years, lactulose has been considered the “bifidus factor”, increasing fecal *Bifidobacterium* counts [68]. A number of subsequent studies have confirmed increased intestinal content of *Bifidobacterium* [69–75] and *Lactobacillus* [69, 72] in human stool samples upon lactulose exposure. Those studies relied on conventional microbiological techniques and did not address the overall composition of the intestinal microbiota. Furthermore, in contrast to our study, lower dosages of lactulose (5–20 g) and longer exposure times (4–8 weeks) were used, potentially explaining differences to our results. In fact, effects on *Bifidobacterium* stool densities were weaker and not significantly different from controls in an 8-day study [76]. However, short-term effects of lactulose on the intestinal microbiota have not been tested previously. No “bifidus factor” effects were observed in our study and differences in the time of lactulose exposure might explain this discrepancy.

Fermentation of lactulose is not limited to *Lactobacillus* and *Bifidobacterium*. Glucosidases and bacterial transporters for lactulose are abundant in a large number of intestinal bacteria including *Cronobacter*, *Enterococcus*, *Escherichia*, *Klebsiella*, *Pseudomonas*, and *Streptococcus* spp., which were able to grow on lactulose as the sole carbon source [13]. One recent study addressed effects of feeding mice with lactulose for two weeks: The authors noted a decrease in

pH and an increase in short chain fatty acids in the colon. In the same mice, an increase in Proteobacteria and Actinobacteria including *Bifidobacterium* and a decrease in Firmicutes was observed. An increase in *Helicobacter* and *Akkermansia* spp. content was interpreted to be secondary to increased mucin production. Similar to our study, no increase in *E. coli* content was observed [77]. These changes were accompanied by an increase in *Bifidobacterium*, *Lactobacillus* spp. and Enterobacteriaceae. In our study, we found a remarkable stability of the microbiota upon lactulose perturbation with 50 g lactulose resulting in diarrhea in >80% of participants. In fact, day to day variation in the control group were as big as variations in the experimental group and only approximately 3% of the variance can be explained by lactulose treatment (not shown).

Effects of lactulose on growth of intestinal pathogens are controversial. *In vitro* co-cultures of human intestinal *Lactobacillus* isolates with *E. coli*, *Salmonella enteritidis* or *Vibrio cholerae*, demonstrated reduced growth of the enteric bacterial pathogens [78]. Case series also demonstrated therapeutic effects of lactulose treatment for human carriers of non-typhoid *Salmonellae* [79] and a temporary reduction of *Shigella* excretion in carriers [80]. Application of lactulose was suggested to decrease the number of urinary tract infections [81]. However, in a rat model of *Salmonella enteritidis* infection, lactulose inhibited *S. Typhimurium* colonization, but stimulated bacterial translocation and intestinal inflammation [82].

In our study, we were aiming to establish an experimental model for improved growth conditions for Enterobacteriaceae. *In vivo* data from our group with a murine colitis model suggested that the initial growth of *S. Typhimurium* within the first day depended on the presence of enzymes enabling hydrogen utilization [18]. Better growth conditions for *S. Typhimurium* would suggest a higher susceptibility for bacterial infections upon lactulose exposure. This might be relevant for patients with liver cirrhosis who are highly susceptible to bacterial infections [83] and for whom lactulose remains the first-line drug for treatment and prevention of hepatic encephalopathy [9].

In our study, we did not find an increase in *E. coli* levels one day after lactulose application even though *E. coli* could potentially benefit from H<sub>2</sub> produced upon lactulose ingestion. In agreement with our results, no clinical data support an increased risk of Enterobacteriaceae infections upon lactulose exposure. One reason for this could be that intrinsic *E. coli* are part of metabolic networks in biofilms in the large intestine and might still depend on metabolic contributions from other bacteria (according to the "Restaurant" hypothesis) [84], which limits any stimulatory effects of H<sub>2</sub> and further studies for the metabolization of lactulose in the intestine are warranted.

In summary, exposure with a single lactulose dose leads to diarrhea in >80% of healthy individuals but not to an increase in *E. coli* CFU and no microbiota shifts. A single lactulose dose is therefore unlikely to significantly improve growth conditions of H<sub>2</sub>-consuming enteropathogens such as *S. Typhimurium* in the human gut.

## Supporting information

**S1 File. Study protocol.** Detailed protocol of all study procedures including lactulose and sucrose treatment.  
(DOCX)

**S2 File. CONSORT statement.**  
(DOC)

**S1 Fig. Relationship of bacterial counts with hydrogen levels and clinical symptoms.** (A) Correlation analysis between maximum (max) hydrogen levels and bacterial loads within feces

collected one day after treatment. (B) Correlation analysis between bacterial loads within feces collected one day after treatment and the number of defecations during 6 hours following lactulose or sucrose ingestion. (C) Correlation between bacterial loads within feces collected one day after treatment and Bristol stool scale during 6 hours following lactulose or sucrose ingestion.  $p > 0.05$  = not significant;  $**p < 0.01$  = significant; Spearman R correlation. Dashed lines = detection limit.

(EPS)

**S2 Fig. The abundance of Enterobacteriaceae (family) and *Escherichia-Shigella* (genus) do not differ between time points and treatments.** (A) Taxonomic annotation of five OTUs of the family of Enterobacteriaceae are shown, one of which belongs to the genus *Escherichia/Shigella*, which had a 100% sequence match to *Escherichia coli*. (B) The relative abundance of members of the genus *Escherichia-Shigella* (striped, upper right part) and the family Enterobacteriaceae (lower left part) show no significant ( $p < 0.05$ ) differences according to treatment (lactulose vs. sucrose) or between time points (paired Wilcoxon test, with FDR correction). The numbers in the boxes are FDR corrected p-values for the group comparisons. The color key indicates the difference [%] in mean relative abundance (percentage of all bacteria) between two groups  $r_i - c_j$ , where  $r_i$  represents the mean relative abundance of the group identified by the row label and  $c_j$  the mean relative abundance of the group identified by the column label. Difference in abundance is expressed as mean difference of the percentages.

(PDF)

**S1 Table Detailed quantification of symptoms by individual participants.**

(DOCX)

**S2 Table. Antibiotic resistances of isolated *E. coli* strains.** Dark grey indicates resistance against the respective antibiotics, medium grey indicates intermediate resistance against the tested antibiotic (this depends on the antibiotic) and light grey means susceptibility to the antibiotic.

(DOCX)

## Author Contributions

**Conceptualization:** Sandra Y. Wotzka, Markus Kreuzer, Lisa Maier, Mirjam Zünd, Markus Schlumberger, Mark Fox, Gerhard Rogler, Luc Biedermann, Michael Scharl, Shinichi Sunagawa, Wolf-Dietrich Hardt, Benjamin Misselwitz.

**Data curation:** Sandra Y. Wotzka, Markus Kreuzer, Lisa Maier, Mirjam Zünd, Bidong Nguyen, Henriette Heinrich, Michael Scharl, Shinichi Sunagawa, Benjamin Misselwitz.

**Formal analysis:** Sandra Y. Wotzka, Lisa Maier, Mirjam Zünd, Markus Schlumberger, Bidong Nguyen, Benjamin Misselwitz.

**Funding acquisition:** Mark Fox, Benjamin Misselwitz.

**Investigation:** Markus Kreuzer, Markus Schlumberger, Daniel Pohl, Gerhard Rogler, Luc Biedermann, Michael Scharl.

**Methodology:** Henriette Heinrich, Gerhard Rogler, Michael Scharl, Shinichi Sunagawa, Wolf-Dietrich Hardt, Benjamin Misselwitz.

**Project administration:** Lisa Maier, Bidong Nguyen, Mark Fox, Benjamin Misselwitz.

**Resources:** Daniel Pohl, Michael Scharl, Wolf-Dietrich Hardt.



**Software:** Benjamin Misselwitz.

**Supervision:** Markus Schlumberger, Mark Fox, Gerhard Rogler, Luc Biedermann.

**Validation:** Benjamin Misselwitz.

**Visualization:** Markus Kreuzer, Benjamin Misselwitz.

**Writing – original draft:** Sandra Y. Wotzka, Markus Kreuzer, Lisa Maier, Markus Schlumberger, Bidong Nguyen, Mark Fox, Gerhard Rogler, Luc Biedermann, Michael Scharl, Shinichi Sunagawa, Wolf-Dietrich Hardt, Benjamin Misselwitz.

**Writing – review & editing:** Sandra Y. Wotzka, Markus Kreuzer, Lisa Maier, Mirjam Zünd, Markus Schlumberger, Bidong Nguyen, Daniel Pohl, Henriette Heinrich, Gerhard Rogler, Luc Biedermann, Michael Scharl, Shinichi Sunagawa, Wolf-Dietrich Hardt, Benjamin Misselwitz.

## References

1. Stecher B, Hardt WD. Mechanisms controlling pathogen colonization of the gut. *Curr Opin Microbiol*. 2011; 14(1):82–91. <https://doi.org/10.1016/j.mib.2010.10.003> PMID: [21036098](#).
2. Stecher B, Berry D, Loy A. Colonization resistance and microbial ecophysiology: using gnotobiotic mouse models and single-cell technology to explore the intestinal jungle. *FEMS Microbiol Rev*. 2013; 37(5):793–829. <https://doi.org/10.1111/1574-6976.12024> PMID: [23662775](#).
3. Pavia AT, Shipman LD, Wells JG, Puhf ND, Smith JD, McKinley TW, et al. Epidemiologic evidence that prior antimicrobial exposure decreases resistance to infection by antimicrobial-sensitive *Salmonella*. *J Infect Dis*. 1990; 161(2):255–60. PMID: [2299207](#).
4. Barthel M, Hapfelmeier S, Quintanilla-Martinez L, Kremer M, Rohde M, Hogardt M, et al. Pretreatment of mice with streptomycin provides a *Salmonella enterica* serovar Typhimurium colitis model that allows analysis of both pathogen and host. *Infect Immun*. 2003; 71(5):2839–58. <https://doi.org/10.1128/IAI.71.5.2839-2858.2003> PMID: [12704158](#); PubMed Central PMCID: PMC153285.
5. Bohnhoff M, Drake BL, Miller CP. Effect of streptomycin on susceptibility of intestinal tract to experimental *Salmonella* infection. *Proc Soc exp Biol, NY*. 1954; 86:132–37.
6. Schuster-Wolf-Bühning R, Fischer L, Hinrichs J. Production and physiological action of the disaccharide lactulose. *International Dairy Journal*. 2010; 20(11):731–41.
7. Panesar PS, Kumari S. Lactulose: production, purification and potential applications. *Biotechnol Adv*. 2011; 29(6):940–8. <https://doi.org/10.1016/j.biotechadv.2011.08.008> PMID: [21856402](#).
8. Treatments for Constipation: A Review of Systematic Reviews. CADTH Rapid Response Reports. Ottawa (ON)2014.
9. Sharma P, Sharma BC. Disaccharides in the treatment of hepatic encephalopathy. *Metab Brain Dis*. 2013; 28(2):313–20. <https://doi.org/10.1007/s11011-013-9392-4> PMID: [23456517](#).
10. Rezaie A, Buresi M, Lembo A, Lin H, McCallum R, Rao S, et al. Hydrogen and Methane-Based Breath Testing in Gastrointestinal Disorders: The North American Consensus. *Am J Gastroenterol*. 2017; 112(5):775–84. <https://doi.org/10.1038/ajg.2017.46> PMID: [28323273](#); PubMed Central PMCID: PMC5418558.
11. Hoffmann K, Mossel DA, Korus W, Van De Kamer JH. [Studies on the Mechanism of Action of Lactulose (Beta-Galactoside Action of Lactulose (Beta-Galactosido-Fructose) in the Intestine]. *Klin Wochenschr*. 1964; 42:126–30. PMID: [14152610](#).
12. Cummings JH. Short chain fatty acids in the human colon. *Gut*. 1981; 22(9):763–79. PMID: [7028579](#); PubMed Central PMCID: PMC1419865.
13. Mao B, Li D, Zhao J, Liu X, Gu Z, Chen YQ, et al. In vitro fermentation of lactulose by human gut bacteria. *J Agric Food Chem*. 2014; 62(45):10970–7. <https://doi.org/10.1021/jf503484d> PMID: [25340538](#).
14. Stecher B, Chaffron S, Kappeli R, Hapfelmeier S, Friedrich S, Weber TC, et al. Like will to like: abundances of closely related species can predict susceptibility to intestinal colonization by pathogenic and commensal bacteria. *PLoS Pathog*. 2010; 6(1):e1000711. <https://doi.org/10.1371/journal.ppat.1000711> PMID: [20062525](#); PubMed Central PMCID: PMC2796170.
15. El Kaoutari A, Armougom F, Gordon JI, Raoult D, Henricsson B. The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. *Nat Rev Microbiol*. 2013; 11(7):497–504. <https://doi.org/10.1038/nrmicro3050> PMID: [23748339](#).

16. Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat Rev Microbiol*. 2008; 6(2):121–31. <https://doi.org/10.1038/nrmicro1817> PMID: 18180751.
17. Lamichhane-Khadka R, Frye JG, Porwollik S, McClelland M, Maier RJ. Hydrogen-stimulated carbon acquisition and conservation in *Salmonella enterica* serovar Typhimurium. *J Bacteriol*. 2011; 193(20):5824–32. <https://doi.org/10.1128/JB.05456-11> PMID: 21856852; PubMed Central PMCID: PMC3187206.
18. Maier L, Vyas R, Cordova CD, Lindsay H, Schmidt TS, Brugiroux S, et al. Microbiota-derived hydrogen fuels *Salmonella typhimurium* invasion of the gut ecosystem. *Cell Host Microbe*. 2013; 14(6):641–51. <https://doi.org/10.1016/j.chom.2013.11.002> PMID: 24331462.
19. Maier RJ. Use of molecular hydrogen as an energy substrate by human pathogenic bacteria. *Biochem Soc Trans*. 2005; 33(Pt 1):83–5. <https://doi.org/10.1042/BST0330083> PMID: 15667272.
20. McNorton MM, Maier RJ. Roles of H<sub>2</sub> uptake hydrogenases in *Shigella flexneri* acid tolerance. *Microbiology*. 2012; 158(Pt 8):2204–12. <https://doi.org/10.1099/mic.0.058248-0> PMID: 22628482; PubMed Central PMCID: PMC3542139.
21. Zbell AL, Maier RJ. Role of the Hya hydrogenase in recycling of anaerobically produced H<sub>2</sub> in *Salmonella enterica* serovar Typhimurium. *Appl Environ Microbiol*. 2009; 75(5):1456–9. <https://doi.org/10.1128/AEM.02064-08> PMID: 19114523; PubMed Central PMCID: PMC2648180.
22. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, et al. Linking long-term dietary patterns with gut microbial enterotypes. *Science*. 2011; 334(6052):105–8. <https://doi.org/10.1126/science.1208344> PMID: 21885731; PubMed Central PMCID: PMC3368382.
23. Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, et al. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME J*. 2011; 5(2):220–30. <https://doi.org/10.1038/ismej.2010.118> PMID: 20686513; PubMed Central PMCID: PMC3105703.
24. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature*. 2014; 505(7484):559–63. <https://doi.org/10.1038/nature12820> PMID: 24336217; PubMed Central PMCID: PMC3957428.
25. David LA, Materna AC, Friedman J, Campos-Baptista MI, Blackburn MC, Perrotta A, et al. Host lifestyle affects human microbiota on daily timescales. *Genome Biol*. 2014; 15(7):R89. <https://doi.org/10.1186/gb-2014-15-7-r89> PMID: 25146375; PubMed Central PMCID: PMC4405912.
26. Wasfy M, Oyoyo B, Elgindy A, Churilla A. Comparison of preservation media for storage of stool samples. *J Clin Microbiol*. 1995; 33(8):2176–8. PMID: 7559972; PubMed Central PMCID: PMCPMC228359.
27. Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, Walsh F, et al. Tackling antibiotic resistance: the environmental framework. *Nat Rev Microbiol*. 2015; 13(5):310–7. <https://doi.org/10.1038/nrmicro3439> PMID: 25817583.
28. Shan M, Gentile M, Yeiser JR, Walland AC, Bornstein VU, Chen K, et al. Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. *Science*. 2013; 342(6157):447–53. Epub 2013/09/28. <https://doi.org/10.1126/science.1237910> PMID: 24072822; PubMed Central PMCID: PMCPMC4005805.
29. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement M100-S24. CLSI Clinical and Laboratory Standards Institute. 2014.
30. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol*. 1991; 173(2):697–703. PMID: 1987160; PubMed Central PMCID: PMC207061.
31. Stecher B, Robbani R, Walker AW, Westendorf AM, Barthel M, Kremer M, et al. *Salmonella enterica* serovar typhimurium exploits inflammation to compete with the intestinal microbiota. *PLoS Biol*. 2007; 5(10):2177–89. <https://doi.org/10.1371/journal.pbio.0050244> PMID: 17760501; PubMed Central PMCID: PMC1951780.
32. Johnson M, Zaretskaya I, Raytselis Y, Merezukh Y, McGinnis S, Madden TL. NCBI BLAST: a better web interface. *Nucleic Acids Res*. 2008; 36(Web Server issue):W5–9. <https://doi.org/10.1093/nar/gkn201> PMID: 18440982; PubMed Central PMCID: PMC2447716.
33. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*. 2004; 32(5):1792–7. <https://doi.org/10.1093/nar/gkh340> PMID: 15034147; PubMed Central PMCID: PMC390337.
34. Huson DH, Bryant D. Application of phylogenetic networks in evolutionary studies. *Mol Biol Evol*. 2006; 23(2):254–67. <https://doi.org/10.1093/molbev/msj030> PMID: 16221896.
35. Apprill A, McNally S, Parsons R, Weber L. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *AQUATIC MICROBIAL ECOLOGY*. 2015; 75:129–37. <https://doi.org/10.3354/ame01753>



36. Parada AE, Needham DM, Fuhrman JA. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ Microbiol*. 2016; 18(5):1403–14. <https://doi.org/10.1111/1462-2920.13023> PMID: 26271760.
37. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*. 2010; 26(19):2460–1. Epub 2010/08/17. <https://doi.org/10.1093/bioinformatics/btq461> PMID: 20709691.
38. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al. *vegan: Community Ecology Package* 2017 [cited 2018 31.08.2018]. Available from: <https://cran.r-project.org/package=vegan>.
39. Wickham H. *ggplot2: Elegant Graphics for Data Analysis*: Springer-Verlag New York; 2009 [cited 2018 31.08.2018]. Available from: <http://ggplot2.org/>.
40. Faul F, Erdfelder E, Lang AG, Buchner A. G\*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods*. 2007; 39(2):175–91. PMID: 17695343.
41. Portalatin M, Winstead N. Medical management of constipation. *Clin Colon Rectal Surg*. 2012; 25(1):12–9. <https://doi.org/10.1055/s-0032-1301754> PMID: 23449608; PubMed Central PMCID: PMC3348737.
42. Wirz S, Nadstawek J, Elsen C, Junker U, Wartenberg HC. Laxative management in ambulatory cancer patients on opioid therapy: a prospective, open-label investigation of polyethylene glycol, sodium picosulphate and lactulose. *Eur J Cancer Care (Engl)*. 2012; 21(1):131–40. <https://doi.org/10.1111/j.1365-2354.2011.01286.x> PMID: 21880080.
43. Chassagne P, Ducrotte P, Garnier P, Mathiex-Fortunet H. Tolerance and Long-Term Efficacy of Polyethylene Glycol 4000 (Forlax(R)) Compared to Lactulose in Elderly Patients with Chronic Constipation. *J Nutr Health Aging*. 2017; 21(4):429–39. <https://doi.org/10.1007/s12603-016-0762-6> PMID: 28346570.
44. Huang CH, Lin JS, Li TC, Lee SC, Wang HP, Lue HC, et al. Comparison of a Chinese Herbal Medicine (CCH1) and Lactulose as First-Line Treatment of Constipation in Long-Term Care: A Randomized, Double-Blind, Double-Dummy, and Placebo-Controlled Trial. *Evid Based Complement Alternat Med*. 2012; 2012:923190. <https://doi.org/10.1155/2012/923190> PMID: 22474530; PubMed Central PMCID: PMC3313604.
45. Emami Alorizi SM, Fattahi MR, Saghebi SA, Salehi A, Rezaeizadeh H, Nimrouzi M, et al. Assessment of the impacts of traditional Persian medical schemes and recommendations on functional chronic constipation compared to a classic medicine lactulose, a randomized clinical trial. *J Complement Integr Med*. 2015; 12(4):325–31. <https://doi.org/10.1515/jcim-2015-0013> PMID: 26214609.
46. Lee-Robichaud H, Thomas K, Morgan J, Nelson RL. Lactulose versus Polyethylene Glycol for Chronic Constipation. *Cochrane Database Syst Rev*. 2010;(7):CD007570. <https://doi.org/10.1002/14651858.CD007570.pub2> PMID: 20614462.
47. Gordon M, MacDonald JK, Parker CE, Akobeng AK, Thomas AG. Osmotic and stimulant laxatives for the management of childhood constipation. *Cochrane Database Syst Rev*. 2016;(8):CD009118. <https://doi.org/10.1002/14651858.CD009118.pub3> PMID: 27531591.
48. Taylor RR, Guest JF. The cost-effectiveness of macrogol 3350 compared to lactulose in the treatment of adults suffering from chronic constipation in the UK. *Aliment Pharmacol Ther*. 2010; 31(2):302–12. <https://doi.org/10.1111/j.1365-2036.2009.04191.x> PMID: 19886948.
49. Maydeo A. Lactitol or lactulose in the treatment of chronic constipation: result of a systematic. *J Indian Med Assoc*. 2010; 108(11):789–92. PMID: 21510584.
50. Triantafyllou K, Chang C, Pimentel M. Methanogens, methane and gastrointestinal motility. *J Neurogastroenterol Motil*. 2014; 20(1):31–40. <https://doi.org/10.5056/jnm.2014.20.1.31> PMID: 24466443; PubMed Central PMCID: PMC3895606.
51. Krajmalnik-Brown R, Ilhan ZE, Kang DW, DiBaise JK. Effects of gut microbes on nutrient absorption and energy regulation. *Nutr Clin Pract*. 2012; 27(2):201–14. <https://doi.org/10.1177/0884533611436116> PMID: 22367888; PubMed Central PMCID: PMC3601187.
52. Gasbarrini A, Corazza GR, Gasbarrini G, Montalto M, Di Stefano M, Basilisco G, et al. Methodology and indications of H2-breath testing in gastrointestinal diseases: the Rome Consensus Conference. *Aliment Pharmacol Ther*. 2009; 29 Suppl 1:1–49. <https://doi.org/10.1111/j.1365-2036.2009.03951.x> PMID: 19344474.
53. Levitt MD. Volume and composition of human intestinal gas determined by means of an intestinal wash-out technic. *N Engl J Med*. 1971; 284(25):1394–8. <https://doi.org/10.1056/NEJM197106242842502> PMID: 5578321.
54. Bratten JR, Spanier J, Jones MP. Lactulose breath testing does not discriminate patients with irritable bowel syndrome from healthy controls. *Am J Gastroenterol*. 2008; 103(4):958–63. <https://doi.org/10.1111/j.1572-0241.2008.01785.x> PMID: 18371134.

55. Lasa J, Peralta D, Dima G, Novillo A, Besasso H, Soifer L. Comparison of abdominal bloating severity between Irritable Bowel Syndrome patients with high and low levels of breath hydrogen excretion in a lactulose breath test. *Rev Gastroenterol Mex*. 2012; 77(2):53–7. <https://doi.org/10.1016/j.rgm.2012.02.002> PMID: 22658548.
56. Lee KN, Lee OY, Koh DH, Sohn W, Lee SP, Jun DW, et al. Association between symptoms of irritable bowel syndrome and methane and hydrogen on lactulose breath test. *J Korean Med Sci*. 2013; 28(6):901–7. <https://doi.org/10.3346/jkms.2013.28.6.901> PMID: 23772156; PubMed Central PMCID: PMC3678008.
57. Youn YH, Park JS, Jahng JH, Lim HC, Kim JH, Pimentel M, et al. Relationships among the lactulose breath test, intestinal gas volume, and gastrointestinal symptoms in patients with irritable bowel syndrome. *Dig Dis Sci*. 2011; 56(7):2059–66. <https://doi.org/10.1007/s10620-011-1569-2> PMID: 21240630.
58. Zhu Y, Zheng X, Cong Y, Chu H, Fried M, Dai N, et al. Bloating and distention in irritable bowel syndrome: the role of gas production and visceral sensation after lactose ingestion in a population with lactase deficiency. *Am J Gastroenterol*. 2013; 108(9):1516–25. <https://doi.org/10.1038/ajg.2013.198> PMID: 23917444.
59. Chatterjee S, Park S, Low K, Kong Y, Pimentel M. The degree of breath methane production in IBS correlates with the severity of constipation. *Am J Gastroenterol*. 2007; 102(4):837–41. <https://doi.org/10.1111/j.1572-0241.2007.01072.x> PMID: 17397408.
60. Kunkel D, Basseri RJ, Makhani MD, Chong K, Chang C, Pimentel M. Methane on breath testing is associated with constipation: a systematic review and meta-analysis. *Dig Dis Sci*. 2011; 56(6):1612–8. <https://doi.org/10.1007/s10620-011-1590-5> PMID: 21286935.
61. Simon GL, Gorbach SL. Intestinal flora in health and disease. *Gastroenterology*. 1984; 86(1):174–93. Epub 1984/01/01. PMID: 6357937.
62. Marteau P, Pochart P, Dore J, Bera-Maillet C, Bernalier A, Corthier G. Comparative study of bacterial groups within the human cecal and fecal microbiota. *Appl Environ Microbiol*. 2001; 67(10):4939–42. Epub 2001/09/26. <https://doi.org/10.1128/AEM.67.10.4939-4942.2001> PMID: 11571208; PubMed Central PMCID: PMC393255.
63. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. *Science*. 2005; 308(5728):1635–8. Epub 2005/04/16. <https://doi.org/10.1126/science.1110591> PMID: 15831718; PubMed Central PMCID: PMC1395357.
64. Berg RD. The indigenous gastrointestinal microflora. *Trends Microbiol*. 1996; 4(11):430–5. Epub 1996/11/01. PMID: 8950812.
65. Todar K. Online Textbook of Bacteriology—The Normal Bacterial Flora of Humans 2018 [cited 2018 28.08.2018]. Available from: [www.textbookofbacteriology.net](http://www.textbookofbacteriology.net)
66. Gibson GR, Probert HM, Loo JV, Rastall RA, Roberfroid MB. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr Res Rev*. 2004; 17(2):259–75. <https://doi.org/10.1079/NRR200479> PMID: 19079930.
67. Slavin J. Fiber and prebiotics: mechanisms and health benefits. *Nutrients*. 2013; 5(4):1417–35. <https://doi.org/10.3390/nu5041417> PMID: 23609775; PubMed Central PMCID: PMC3705355.
68. Petuely F. [Lactobacillus bifidus flora produced in artificially-fed infants by bifidogenic substances (bifidus factor)]. *Z Kinderheilkd*. 1957; 79(2):174–9. PMID: 13531346.
69. Ballongue J, Schumann C, Quignon P. Effects of lactulose and lactitol on colonic microflora and enzymatic activity. *Scand J Gastroenterol Suppl*. 1997; 222:41–4. <https://doi.org/10.1080/00365521.1997.11720716> PMID: 9145445.
70. Vanhoutte T, De Preter V, De Brandt E, Verbeke K, Swings J, Huys G. Molecular monitoring of the fecal microbiota of healthy human subjects during administration of lactulose and *Saccharomyces boulardii*. *Appl Environ Microbiol*. 2006; 72(9):5990–7. <https://doi.org/10.1128/AEM.00233-06> PMID: 16957220; PubMed Central PMCID: PMC1563651.
71. De Preter V, Vanhoutte T, Huys G, Swings J, Rutgeerts P, Verbeke K. Effect of lactulose and *Saccharomyces boulardii* administration on the colonic urea-nitrogen metabolism and the bifidobacteria concentration in healthy human subjects. *Aliment Pharmacol Ther*. 2006; 23(7):963–74. <https://doi.org/10.1111/j.1365-2036.2006.02834.x> PMID: 16573799.
72. Tayebi-Khosroshahi H, Habibzadeh A, Niknafs B, Ghotaslou R, Yeganeh Sefidan F, Ghojzadeh M, et al. The effect of lactulose supplementation on fecal microflora of patients with chronic kidney disease: a randomized clinical trial. *J Renal Inj Prev*. 2016; 5(3):162–7. <https://doi.org/10.15171/jrip.2016.34> PMID: 27689115; PubMed Central PMCID: PMC5040005.
73. Tuohy KM, Ziemer CJ, Klinder A, Knobel Y, Pool-Zobel BL, Gibson GR. A Human Volunteer Study to Determine the Prebiotic Effects of Lactulose Powder on Human Colonic Microbiota. *Microbial Ecology in Health and Disease*. 2002; 14:165.

74. Bouhnik Y, Attar A, Joly FA, Riottot M, Dyard F, Flourie B. Lactulose ingestion increases faecal bifidobacterial counts: a randomised double-blind study in healthy humans. *Eur J Clin Nutr*. 2004; 58(3):462–6. <https://doi.org/10.1038/sj.ejcn.1601829> PMID: 14985684.
75. Bouhnik Y, Neut C, Raskine L, Michel C, Riottot M, Andrieux C, et al. Prospective, randomized, parallel-group trial to evaluate the effects of lactulose and polyethylene glycol-4000 on colonic flora in chronic idiopathic constipation. *Aliment Pharmacol Ther*. 2004; 19(8):889–99. <https://doi.org/10.1111/j.1365-2036.2004.01918.x> PMID: 15080850.
76. Bouhnik Y, Raskine L, Simoneau G, Vicaut E, Neut C, Flourie B, et al. The capacity of nondigestible carbohydrates to stimulate fecal bifidobacteria in healthy humans: a double-blind, randomized, placebo-controlled, parallel-group, dose-response relation study. *Am J Clin Nutr*. 2004; 80(6):1658–64. <https://doi.org/10.1093/ajcn/80.6.1658> PMID: 15585783.
77. Mao B, Li D, Ai C, Zhao J, Zhang H, Chen W. Lactulose Differently Modulates the Composition of Luminal and Mucosal Microbiota in C57BL/6J Mice. *J Agric Food Chem*. 2016; 64(31):6240–7. <https://doi.org/10.1021/acs.jafc.6b02305> PMID: 27438677.
78. Drago L, Gismondo MR, Lombardi A, de Haen C, Gozzini L. Inhibition of in vitro growth of enteropathogens by new *Lactobacillus* isolates of human intestinal origin. *FEMS Microbiol Lett*. 1997; 153(2):455–63. PMID: 9271875.
79. Hoffmann K. Behandlung von gesunden Salmonellen-ausscheidern mit lactulose. *Dtsch Med Wochenschr* 1975; 100:1429. <https://doi.org/10.1055/s-0028-1106401> PMID: 238823
80. Levine MM, Hornick RB. Lactulose therapy in *Shigella* carrier state and acute dysentery. *Antimicrob Agents Chemother*. 1975; 8(5):581–4. PubMed Central PMCID: PMC429425. PMID: 2098
81. Battle M, Martin T, Fulton J. Lactulose may help prevent urinary tract infections. *BMJ*. 2001; 323(7318):936. PMID: 11668146; PubMed Central PMCID: PMC1121453.
82. Bovee-Oudenhoven IM, ten Bruggencate SJ, Lettink-Wissink ML, van der Meer R. Dietary fructo-oligosaccharides and lactulose inhibit intestinal colonisation but stimulate translocation of salmonella in rats. *Gut*. 2003; 52(11):1572–8. PMID: 14570725; PubMed Central PMCID: PMC1773861.
83. Ascione T, Di Flumeri G, Boccia G, De Caro F. Infections in patients affected by liver cirrhosis: an update. *Infez Med*. 2017; 25(2):91–7. PMID: 28603226.
84. Conway T, Cohen PS. Commensal and Pathogenic *Escherichia coli* Metabolism in the Gut. *Microbiol Spectr*. 2015; 3(3). <https://doi.org/10.1128/microbiolspec.MBP-0006-2014> PMID: 26185077; PubMed Central PMCID: PMC4510460.